The gigas effect: A reliable predictor of ploidy? Case studies in *Oxalis*

by
Frederik Willem Becker

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Supervisors: Prof. Léanne L.Dreyer, Dr. Kenneth C. Oberlander, Dr. Pavel Trávníček

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Abstract

Whole Genome Duplication (WGD), or polyploidy is an important evolutionary process, but literature is divided over its long-term evolutionary potential to generate diversity and lead to lineage divergence. WGD often causes major phenotypic changes in polyploids, of which the most prominent is the Gigas effect. The Gigas effect refers to the enlargement of plant cells due to their increased amount of DNA, causing plant organs to enlarge as well. This enlargement has been associated with fitness advantages in polyploids, enabling them to successfully establish and persist, eventually causing speciation. Using *Oxalis* as a study system, I examine whether *Oxalis* polyploids exhibit the Gigas effect using 24 species across the genus from the *Oxalis* living research collection at the Stellenbosch University Botanical Gardens, Stellenbosch. Given that the Gigas effect also holds great potential to increase a polyploid's competitive ability, and as a result, invasiveness, I also tested for the Gigas effect in 15 traits and WGD-associated increased self-fertilization and bulbil production in the weedy species *O. purpurea*. Using known correlates of the Gigas effect (stomata length, epidermal cell area and pollen grain diameter) I show that *Oxalis* polyploids display a very inconsistent and small Gigas effect – contrary to that predicted from the literature. With extensive sampling across 20 populations of *O. purpurea* in its native range, I show a similar pattern for stomata length, pollen grain diameter and epidermal cell area in this species. In addition, I found a large decrease in effect size of polyploidy and substantial variation across traits in 12 further leaf and flower traits studied. *O. purpurea* showed very high levels of self-incompatibility among both diploids and polyploids, but polyploids produced significantly more and heavier bulbils than diploids. Overall, these results revealed a very small and inconsistent Gigas effect among *Oxalis* polyploids. There is, however, an association between polyploidy and invasive potential, using bulbil production as a proxy for invasiveness. Polyploid success and persistence in *Oxalis* could be as a result of a temporary initial Gigas effect upon formation, which later becomes diluted through local adaptation.

Opsomming

Heel Genoom Duplisering (HGD), of polyploidie, is ‘n belangrike evolusionêre proses, maar die literatuur is verdeeld oor die lang termyn evolusionêre potensiaal van HGD om spesies te vorm. HGD veroorsaak opvallende veranderinge in fenotipiese eienskappe waarvan die mees prominente effek die “Gigas” effek is. Die “Gigas” effek verwys na die vergroting van plant selle as gevolg van meer DNA en, gevolglik, ook groter organe. Hierdie vergroting het ‘n sterk verwantskap met verhoogde fiksheid in polyploiëde wat lei daarnatoe dat hulle gevestigde bevolkings vorm en aanhou voortleef. Dit kan gevolglik lei daarnatoe dat polyploiëde nuwe spesies kan vorm. Ek ondersoek die vraag of polyploiëde betroubaar die “Gigas” effek toon deur gebruik te maak van 24 spesies in die *Oxalis* genus van die *Oxalis* lewende versameling in die Stellenbosch Universiteit Botaniese Tuine. Gegewe die fikdheids voordele van die “Gigas” effek, is daar ook dikwels eienskappe te vind wat sterk verband hou met indringende eienskappe van plante. Daarom toets ek ook vir die “Gigas” effek in 15 eienskappe en HGD-verwante afbraak in self-onversoenbare genetiese faktore in voortplanting en klonale-bol produksie in die onkruidagtige spesie, *Oxalis purpurea*. Deur gebruik te maak van bekende korrelerende eienskappe van die “Gigas” effek (huidmodjie lengte, epidermale sel oppervlak en stuifmeel korrel diameter) wys ek dat *Oxalis* polyploiëde ‘n baie klein en strydige “Gigas” effek het, teenstrydig met die voorspellings van die literatuur. Met ‘n monsterneming van 20 bevolkings van *O. purpurea* wys ek ook dieselfde patroon in hierdie eienskappe. Verder vind ek ook ‘n geweldige afname in effek-grootte van polyploiëde en aansienlik meer variasie in 12 blaar en blom kemerke waar ek hierdie effek ondersoek het. *O. purpurea* wys baie sterk self-onversoenbaarheid vir beide diploiëde en polyploiëde, maar polyploiëde vervaardig meer en swaarder kloon-bolletjies teennoor diploiëde. Die uitslag van hierdie studies wys dat daar ‘n baie klein en strydige “Gigas” effek in *Oxalis* polyploiëde voorkom. Daar is wel ‘n verwantskap tussen polyploidie en indringendheid in *O. purpurea* deur gebruik te maak van klonale-bol produksie as ‘n toon-kenmerk van indringendheid. Die algehele sukses en voortbestaan van polyploiëde in *Oxalis* mag die uitkoms wees van ‘n tydelik “Gigas” effek onmiddelik na vorming, maar die effek raak later verlore deur plaaslike aanpassing oor geslagte.

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INTRODUCTION

1. Polyploidy

Whole Genome Duplication (WGD), *i.e.* polyploidy, is the phenomenon whereby each unique chromosome set per nucleus is present in more than two (*i.e.* diploid) copies (Frawley and Orr-Weaver 2015). Polyploids can form from one unique chromosome set (through mitotic failure in meristematic tissue), resulting in the formation of autopolyploids (Chen 2007). Autopolyploids and their conspecific diploids often show subtle differences and are difficult to tell apart. Polyploids can also form from two or more genetically distinct chromosome sets (through hybridization), resulting in the formation of auto- or allopolyploids (Liu *et al.* 2017, Vigna *et al.* 2016), depending on the degree of relatedness of the parents. Polyploids with genetically different parents are formed either through a one-step process (fusion of an unreduced egg and an unreduced sperm cell) or a two-step process involving a triploid intermediate (unreduced gamete fusion with a haploid gamete, followed by fusion of a triploid gamete with a haploid gamete) (Ramsey and Schemske 2002). Polyploids occur in almost every taxonomic group on the tree of life, including vertebrates (Van de Peer *et al.*, 2010; Braasch and Postlethwait, 2012 ; Cañestro, 2012), fungi (Hudson and Conant, 2012), ciliates (Aury *et al.*, 2006), many red algae (reviewed by Husband *et al.*2013) and plants, especially ferns and angiosperms (Wendel 2000 , Adams *et al.* 2003, Wood *et al.* 2009). Given its wide taxonomic distribution and occurrence in speciose lineages, polyploidy potentially plays a major role in diversification and increased biological complexity. In this chapter I will briefly review the relevant literature around plant polyploidy and its evolutionary potential to drive speciation.

2. Origin and history

Plant polyploids have been studied for more than 100 years, starting with the first description of a polyploid by De Vries in 1905 in *Oenothera lamarckiana* mut. *gigas* (Onagraceae) (De Vries 1909). The mutant *gigas* showed an overall enlargement of the plant body and was later confirmed to be tetraploid (Lutz 1907, Gates 1909). Even though very little was known about polyploidy back then, suggestions of WGD as driving lineage formation already started to appear, for example, in *Zea mays* L. (Kuwada 1911). More and more polyploid plants were being described, including the formation of a *Primula kewensis* polyploid in Kew Gardens, London in 1905, which was later confirmed to be tetraploid (Digby 1912). Later studies provided more evidence to show that many major crops were polyploid, including wheat, oats,

cotton, tobacco, potato and coffee (McFadden and Sears 1946, Beasley 1940, Goodspeed and Clausen 1928). After successfully forming the first synthetic polyploid (Winkler 1916), Winkler hypothesized that hybridization followed by WGD was a viable means for speciation. His hypothesis was later supported after successful artificial hybridizations in *Nicotiana* L. (Solanaceae) and *Galeopsis* L. (Lamiaceae), and the production of *Raphanobrassica* (Brassicaceae) (Clausen & Goodspeed 1925, Müntzing 1930). Kihara & Ono (1926) first made the distinction between allo- and autopolyploids that, respectively formed via hybridization and not, and thus that hybridization was not the only pathway through which a polyploid species can form.

Müntzing (1936) reviewed the works of early authors, which already showed that WGD impacted phenotypic traits, ecology and genetic incompatibility. On average, polyploids showed larger cells and organs, more robust plant bodies, thicker leaves and larger seeds. Yet some taxa displayed no size increase or smaller traits in polyploids. Furthermore, polyploids also differed in morphology in ways other than size, and often occupied different niches to diploids (Müntzing 1936). They often showed slower cell division, growth rates and germination times (Keeble 1912, Jorgensen 1925). Given the high frequency of polyploids then, and the large impact thereof on morphology, ecology, physiology and genetic isolation, it became apparent that WGD could be significant in the divergence of lineages, ultimately resulting in a new species (Gaiser 1926).

Since its discovery, however, the phenomenon of WGD has caused a divide in the literature regarding its long-term evolutionary role (discussed in Müntzing (1936)). One of the most influential authors in the polyploid literature, and one that contested the idea of polyploidy being an evolutionary driver, was G. Ledyard Stebbins (1906-2000). As summarized in Stebbins (1950, 1971), he argued that polyploids had limited evolutionary potential and very little long-term evolutionary impact (Stebbins 1950), a view which influenced subsequent work such as that of Wagner (1970). He kept this view despite the fact that more and more polyploids were being described, and many synthesized. Essentially, he believed polyploids formed at high rates (estimating that 30-35% of angiosperms formed by historical WGD events) and contributed to variability to some extent, but were only important on short evolutionary time scales, often going extinct. Therefore, WGD was viewed as a process that enabled species groups at certain stages of “biotype depletion” (When genotype variations have not been adapted adequately in a changing environments) to adapt to new environmental conditions that

arise relatively suddenly. This would mean that polyploidy was less important in stable environments, and in diploid species that are widespread and that are rich in ecotypic differentiation. Stebbins (1950, 1971) thought polyploids were formed by a single polyploidization event, exhibiting a high degree of genetic uniformity across individuals, thus rendering polyploids genetically depauperate. If polyploids formed via hybridization, they would only exhibit homeologous variation as opposed to homologous or segregating variation. If a polyploid formed via somatic doubling and a mutation were to arise, its effect would get masked either by the presence of a homeolog locus or multiple alleles (Stebbens 1950, 1971). Finally, he also argued that the fixation of a new mutation would be much slower in polyploids than in diploids.

3. Current views

With the onset of the genomics era, many of Stebbins' views were challenged. Polyploids have been shown to play a prominent role in generating novelty (Levin 1983). Importantly, we also now know that polyploids are formed multiple times and at high rates in nature (Ownbey 1950, Chester *et al.* 2012). WGD has often been associated with major plant radiations (Zhan *et al.* 2016). Although the incidence of polyploidy appears to be low or absent in liverworts, hornworts and cycads, with a large margin of uncertainty (see Roodt *et al.* 2017), it appears frequently in lycophytes, monilophytes and angiosperms (Husband *et al.*, 2013) and has recently been shown also to be more common than previously believed in conifers (Farhat *et al.* 2019). Wood *et al.* (2009) estimated that 15% of speciation events in angiosperms and 31% of speciation events in ferns directly involve polyploidy. Furthermore, WGD is ubiquitous among angiosperms and associated with the formation of major lineages (Van de Peer *et al.*, 2009, 2010).

A WGD event preceded the radiation of all extant angiosperms (Jiao *et al.*, 2011), WGD is associated with the formation of the monocot lineage, and two WGD events, in close temporal succession, appeared early in eudicot evolution (Jiao *et al.*, 2012). There are at least 50 independent WGD events scattered throughout the angiosperm lineage (Cui *et al.*, 2006; Soltis *et al.*, 2009; Van de Peer *et al.*, 2009, 2011). Complete sequencing of *Arabidopsis thaliana* (L.) Heynh., previously thought to be diploid, revealed two or three rounds of duplication in its genome (Vision *et al.*, 2000; Bowers *et al.*, 2003). It has further been shown that WGD is often followed by a burst in species richness in families such as the Brassicaceae, Poaceae and

Solanaceae (Soltis *et al.* 2009, Mandáková *et al.* 2017). It is also associated with an increase in diversity in the Asteraceae, Cleomaceae and Fabaceae (Soltis *et al.*, 2009; Doyle, 2012; Schranz *et al.*, 2012). The tribe Heliophileae, a morphologically diverse lineage in the Brassicaceae that includes the genus *Heliophila* (with *ca*. 90 species), provides another example. In this tribe chromosome number variation largely follows three major lineages, and genomic analyses revealed a Whole Genome Triplication event thought to be linked to its diversification and variation (Mandáková *et al.* 2012, 2017). Other studies have directly linked polyploidy with an increase in biological diversity and complexity (De Smet and Van de Peer, 2012) and there is evidence associating dozens of WGD events with the Cretaceous-Paleogene extinction, suggesting it could have played a role in lineage survival (Fawcett *et al.* 2009, Vanneste *et al.* 2014).

Despite all of this substantial new evidence, the view that polyploids are evolutionary dead-ends is still held by many authors. Polyploidy is not the only mechanism that can introduce variation and novelty. Schranz *et al*. (2012, p. 147) proposed that the "ultimate success of the crown group does not only involve the WGD and novel key traits, but largely subsequent evolutionary phenomena including later migration events, changing environmental conditions and/or differential extinction rates…". It has been shown that polyploids diversify at slower rates, with the majority speciating more slowly than diploids and/or going extinct more often (Mayrose *et al.*, 2011, Arrigo and Barker, 2012). There has also been tentative support (Carta & Peruzzi 2016) for the large genome constraints hypothesis (Knight *et al.* 2005), the idea that lineages with smaller genomes are able to survive across a larger range of climatic niches, whereas lineages with larger genomes are constrained to more intermediate, less extreme environments. Although this does not take into account the occurrence of polyploidy in a lineage per se, it suggests a negative selective pressure on the evolution of large genomes – and therefore also an increase in genome size. However, it has been suggested that polyploidy confers more advantages in unstable environments and that, as a result, polyploids should occur at lower proportions in stable climatic conditions (Stebbins 1971). This may reflect that polyploidy can play a prominent role in diversifying lineages with small genomes in unstable climates, but only until it reaches a certain genome-size threshold, which could be selected against.

Interestingly, Meyers & Levin (2006) showed that the average ploidal level within a lineage can continue to increase to levels seen today, even if there are ecological or physiological

disadvantages to higher ploidy, due to the assumed irreversibility of the WGD process. Knight *et al.* (2005) argued that if there is selection against large genomes in plants, the selection may just be very weak and is "unable to stem the sharp genome size increases perpetuated by fast and powerful forces of DNA addition". Other selective pressures faced by polyploids include those imposed by minority cytotype exclusion (Levin 1975). Theoretical predictions suggest that polyploids should rarely be able to successfully establish in nature (Levin 1983, Fowler and Levin 1984, 2016, Felber 1991, Baack 2005), yet polyploids occur globally (Rice *et al.* 2019), in every major plant lineage. Clearly, we do not yet fully understand the role of polyploidy in evolutionary dynamics.

Although present day literature remains divided on the long-term evolutionary fate of polyploidization (Madlung 2013), it is nevertheless recognized as an important evolutionary process (Müntzing 1936, Darlington 1937, Clausen *et al.* 1945, Löve and Löve 1949, Stebbins 1950, Lewis 1980, Grant 1981, Mable 2003, Gregory & Mable 2005). The ubiquitous occurrence of polyploids, and the close association of WGD with diverging lineages or large radiations, suggests that there may be some selective advantage to being polyploid. A number of features may contribute to the reproductive success, establishment and persistence of a neopolyploid. These include perenniality, and/or a propensity toward apomixis and self-compatibility (van Drunen & Husband 2019). It is also necessary to consider how WGD isolates and differentiates polyploids from diploids, ultimately leading to divergence, if we are to understand the evolutionary impacts and possible benefits of being polyploid.

4. Effects of WGD

Morphological

The effects of WGD on morphology are well documented (reviewed in Knight *et al.* 2005, Doyle & Coate 2019). Using data across 101 angiosperm species, Beaulieu *et al.* (2008) showed there is a significant correlation between WGD and an increase in guard cell length and epidermal cell area, and a decrease in stomatal density. The increase in size, known as the Gigas effect, is discussed in more detail in Section 5. WGD can also affect the relative dimensions of the plant body and organs for example, *Humulus lupus* L. polyploids had thinner, shorter shoots, changed leaf dimensions and areas, shorter flowers, very large lupine glands and significantly heavier cones and spindles (Trojack-Goluch & Skomra 2013). There have been notable changes to flower size, seed coat and seed size in *Nicotiana attenuata* Torr. ex

S.Watson and *Nicotiana obtusifolia* Martens & Galeotti polyploids (Anssour *et al.* 2009). *Acacia mangium* Willd. (4x) differs from two diploid species in terms of flower spike sizes, percentage of male hermaphrodite flowers and the size of the stigma and style (Nghiem *et al.* 2011).

Physiological

WGD has been shown to alter gas exchange rates, gene activity, hormone levels, photosynthetic rates and water balance (Levin 1983, 2002, Warner and Edwards 1993). For example, *Populus tremuloides* Michx. triploids showed greater percentage nitrogen and chlorophyll content and also higher intrinsic water-use efficiency than diploids (Greer *et al.* 2018). Ploidy level has also been correlated with changes in leaf morphology, anatomical traits and physiological processes in six Brassica species (Baker *et al.* 2017). Physiological changes induced by WGD may increase the fitness of a polyploid, for example, polyploidy affected the response to salt stress in polyploid *Robinia* L. species (Wang *et al.* 2013) and photosynthetic response in polyploid *Glycine* J.C. Wendl. species (Coate *et al.* 2012). In another species, *Dendranthema nankingense* (Nakai) Tzvel. tetraploids also had higher tolerance to abiotic stresses than their diploid parents (Liu *et al.* 2011). Higher tolerance of environmental conditions may enable polyploids to outcompete diploids by enhanced growth rate or nutrient and carbon fixation. Changes to physiology may also give rise to pre-adapted states in polyploid enabling them to flourish in introduced or novel environments.

Ecological

As a result of their altered physiological responses, polyploids often occupy different niches to their diploid parents, although the extent to which this occurs varies between taxa (*e.g.*, Martin and Husband 2009, Theodoridis *et al.* 2013, Glennon *et al.* 2014, Harbert *et al.* 2014). Differential niche occupation, and changes in morphology and physiology could also impact the ecological responses of polyploids. Thompson *et al.* (2004) reported that *Heuchera grossulariifolia* Rydb. tetraploids were attacked more frequently by herbivores than diploids. WGD can therefore bring about changes to plant appearances, influencing the ecological community interactions with polyploid plants. Further, Thompson *et al.* (2004) showed that polyploid *Heuchera grossulariifolia* experience a considerable differentiation in the pollinator suites they attracted. Changing pollinator niche is also one way in which polyploids could escape minority cytotype exclusion by diploid cytotypes after WGD. Many studies have shown that WGD alters polyploid habitat use, life history, competitive abilities and interactions with

herbivores, pathogens, pollinators (Oswald and Nuismer, 2007, Thompson and Merg 2008, Arvanitis *et al.* 2010, Boalt *et al.* 2010, Ramsey 2011, Martin and Husband 2013, Strong and Ayres 2013, Ramsey and Ramsey 2014). Ecological interactions could play a complex role in establishment, persistence and extinction of polyploid populations.

Reproductive

Reproductive barriers may be prezygotic (*e.g.* geographic isolation, differences in flowering phenology or pollinator fidelity) or postzygotic (*e.g.* triploid hybrid inviability, inbreeding depression). Changes in morphological features (especially in floral traits) following polyploidization may reinforce the reproductive barriers that prevent mating between cytotypes (Tate *et al.*, 2005). Polyploids are typically considered to be immediately reproductively isolated from their diploid parents by chromosome number, often reinforced by subsequent changes in morphology and physiology. *Chamerion angustifolium* (L.) Scop. (Onagraceae) provides a good example of this (Husband and Sabara 2004), where polyploids were reproductively isolated from diploids by geographic distance, flowering asynchrony, pollinator fidelity, self- pollination and gametic selection. The strongest isolation mechanisms in polyploids were geographic isolation (41%) and pollinator fidelity (44%). A breakdown of genetic incompatibility systems often accompanies WGD, leading to increased selfing rates (Richards, 1997, Barringer 2007). In this way polyploids may increase their chances of successful establishment and escape minority cytotype exclusion. This also partially explains the correlation between invasiveness and WGD (te Beest *et al.* 2012). The same mechanism that enables polyploids to escape minority cytotype exclusion enables polyploids to form viable populations and spread rapidly in introduced ranges.

Genetic

WGD also causes major genetic changes introducing large amounts of variation and novelty in polyploid genomes (Anssour *et al.* 2009). These include changes to genetic structure (Lim *et al.* 2008, Chester *et al.* 2012), gene loss or modification (Wang & Paterson 2011, Kashkush *et al.* 2002), gene expression (Ainouche *et al.* 2012, Hegarty *et al.* 2005) and the formation of new gene functions or division of functions of a gene (Ohno 1970, Lynch & Conery 2000, Lynch & Force 2000, Prince & Pickett 2002, Adams & Wendel 2004, 2005). WGD can lead to an increased adaptive potential in polyploids in particular environments (Ramsey 2011, Selmecki *et al.* 2015, Monnahan *et al.* 2019). This may result in higher rates of beneficial mutations in polyploids and higher rates of relaxed purifying selection on potentially

deleterious mutations (Baduel *et al.* 2019). Novelty in polyploids, however, is not solely attributed to WGD. In fact, ecological and physiological novelty has been linked to epigenetic modifications in polyploids (Osborn *et al.* 2003). Non-additive expression patterns may be generated through chromatin modification, DNA methylation and cis-/trans-acting regulatory interactions (Soltis *et al*. 2014). Importantly, DNA methylation exhibits non-additive patterns following both auto- and allopolyploidization (Salmon *et al.* 2005, Kraitshtein *et al.* 2010, Zhao *et al.* 2011, Lavania *et al.* 2012). Salmon *et al.* (2005) teased apart the role of hybridization and genome duplication per se, indicating that genome merger, and not polyploidy, was largely responsible for non-additive methylation patterns in *Spartina* Schreib. (also see Parisod *et al.* 2009). Yet, alterations to methylation patterns do not always accompany hybridization or polyploidization (Liu *et al.* 2001). Divergent regulatory factors, particularly trans factors acting between parental genomes, also influence gene expression in allopolyploids. These factors are capable of silencing, upregulating or downregulating homeologous loci (Wang *et al.* 2006, Shi *et al.* 2012 and reviewed in Buggs *et al.* 2014). Epigenetic modifications are, however, very seldom carried across multiple generations, limiting their long-term evolutionary impact (see Mendizabal *et al.* 2014).

Evolutionary

Speciation via cladogenesis eventually results in reciprocal monophyly after formation (Rieseberg and Brouillet 1994). Polyphyletic local origins, however, are considered the rule for polyploid species today (Werth *et al.* 1985a, b, Tsigenopoulos *et al.* 2002, Richardson *et al.* 2012). This differs from the mostly single-origin concept of previous authors (Soltis and Soltis 1993, 1999, 2000). Since polyploids of separate origin have been shown to be inter-fertile (Sweigart *et al.*, 2008, Symonds *et al.* 2010), genetic variation may further be increased in polyploids where independently formed lineages form a tokogenic network. This network can incorporate genetic variation from genetically differentiated parental individuals and generate new genotypes through gene flow and recombination (Soltis and Soltis 1999, Tate *et al.* 2005, Soltis *et al.* 2014). However, not all polyploids are equally inter-fertile. Crossing experiments involving *Tragopogon* L. polyploids have shown mixed results when crossing between plants of separate origins (Ownbey and McCollum 1953, Hersch-Green 2012).

The many possible advantageous changes brought on by WGD on genetic, phenotypic, ecological and morphological traits could explain the overall success of plant polyploids. Increased environmental tolerance (Pandit *et al.* 2011) and pre-adaptation to novel

environments as a result of these changes, along with increased levels of self-pollination or clonality may lead to successful establishment and persistence in introduced ranges and eventually lineage diversification.

5. The Gigas effect

Given that consequences of large-scale genomic modifications (such as WGD) can be extensive, linking these changes to their subsequent phenotype is important in the understanding of evolutionary and ecological dynamics (*e.g.*, Otto and Whitton 2000, Flagel and Wendel 2010, Soltis *et al.* 2010). We know that WGD is particularly important in the evolution of polyploid species, as approximately 15% of speciation events in angiosperms (Wood *et al.* 2009) and nearly a quarter of extant plant taxa are polyploid (Barker *et al.* 2016). Perhaps the most prominent phenotypic consequence of duplicating an organisms' DNA is the Gigas effect. The Gigas effect (larger cells and organs) is thought to be brought on by the larger DNA content in every cell (Müntzing 1936, Stebbins 1971), leading to increased cell and consequently organ and plant size. This directional effect is, however, not necessarily always the rule (Otto and Whitton 2000, Vamosi *et al.* 2007). A few exceptions that show different responses have also been documented (Segraves and Thompson 1999, Vamosi *et al.* 2007, Ning *et al.* 2009, Trojak- Goluch and Skomra 2013). Porturas *et al.* (2019) showed that these exceptions are in the minority and that, on average, polyploids do tend towards significantly larger cells and organs, with effect sizes in the range of 20-25% larger in polyploids. Importantly, the study also provided evidence that the effect size of WGD remains consistent across traits and measurement scales.

Having larger cells can impact physiological processes in polyploids. Larger cells take longer to divide (Bennett 1987, Francis *et al.* 2008) and can cause slower growth rates, differences in gaseous exchange, changes in photosynthetic rate and salt stress tolerance (as discussed above). Although slower growth rates and associated metabolic processes might not always increase fitness benefits, it might confer some advantages in environments where polyploids do establish and persist.

Physiological changes may also affect ecology as a result of the Gigas effect. Slower growth rates can lead to delayed flowering phenology or changed pollinator interactions (*i.e.* larger flowers and novel compounds that are more attractive and/or colours change pollinator niche

altogether). Yet there is considerable variation in polyploid phenotypes (e.g., Vamosi *et al.* 2007). This variation is probably caused by post-WGD adaptation, where phenotypes change over generations (Butterfass 1987, Oswald and Nuismer 2011, Ramsey 2011, Husband *et al.* 2016). This highlights the need to study polyploid phenotypes in depth and over time, especially neopolyploids. Despite such variation, the Gigas effect could still be a strong predictor for most polyploid species with a consistent effect size across traits (Porturas *et al.* 2019). The Gigas effect may therefore be a useful tool in uncovering polyploids in the field, as well as explaining their persistence and success.

WGD has been linked with increased invasiveness of species (Pandit *et al.* 2006, 2011). Since polyploids often experience a breakdown of genetic incompatibility systems and may show increased rates of vegetative clonality (Van Drunen & Husband 2019), polyploids may increase propagule pressure when introduced into a new environment. Given the potential cascading effect of the Gigas effect on polyploid morphology, physiology and ecology, there might be a strong relationship between the Gigas effect and invasiveness. The Gigas effect may contribute to the production of more propagules though physiological and metabolic changes or it could enhance the establishment success of polyploid individuals through morphological and physiological effects, increasing survival rates and establishment of clonal propagules in an introduced range.

6. Study system

Oxalis includes *ca*.500 species globally (Lourteig 1994, 1995, 2000). The genus is well represented (*ca*.230 species) in southern Africa, with the vast majority of species endemic to the Greater Cape Floristic Region (GCFR), where it is also the largest geophytic genus. In contrast to most other Cape lineages and contrary to predictions of polyploid abundance in stable climates (Stebbins 1971, Oberlander *et al.* 2016), *Oxalis* has a very large number of polyploids both inside the GCFR (Krejčíková *et al.* 2013 a, b, c) and in the New World (De Azkue 2000, Vaio *et al.* 2016, Luo *et al.* 2006, Emshwiller *et al.* 2009 and Emshwiller 2002). A large number of GCFR *Oxalis* species include extensive polyploid series, often with many cytotypes and also occurring as established populations (*e.g*. in species such as *O. purpurea* L., *O. flava* L. and *O. obtusa* Jacq.). The genus is also morphologically very variable, and species often consist of large species complexes (Salter 1944). Although the possibility that this morphological variation may be linked to different cytotypes has not been studied extensively,

Krejčíková et al. (2013 a, b) found partial correlation between cytotype and environmental parameters (vegetation type and precipitation) in *O. obtusa*, suggesting that polyploidy may drive niche differentiation in *Oxalis* polyploids and contribute to the morphological variability observed in this genus.

Given the prominence of the Gigas effect in polyploids, it may be a reliable predictor of *Oxalis* polyploids and may explain, at least in part, their persistence and success in the GCFR and elsewhere. The link between polyploidy and invasiveness also renders *Oxalis* an interesting study system, given the aggressive weeds included in the genus. *Oxalis pes-caprae* L., for example, is a GCFR-native, but also a globally invasive weed with prominent invasions in Europe, Australia, South Africa (in its native range) and North America (Randall 2012, Sanz Elorza *et al.* 2004). Only diploid, triploid and tetraploid cytotypes are known from unequivocally indigenous populations of this species. In contrast, only tetraploid and pentaploid plants are known from the invaded range (Krejčíková *et al.* 2013 (c)). It has also been observed that, among South African *Oxalis*, species with multiple ploidy levels and wide geographic distributions appear to be the weediest (Krejčíková *et al.* 2013 c).

This is also the case for *Oxalis purpurea* L., an indigenous GCFR species, which have become invasive (Produces reproductive offspring in areas distant from sites of introduction (Richardson *et al.* 2000)) in several parts of the world, with prominent invasions in Australia (Rozefelds *et al.* 1999, Cuevas *et al.* 2004, Paynter *et al.* 1968), the Mediterranean basin, Algeria and California (Randall 2012, Sanz Elorza *et al.* 2004, Randall 2007). It displays weedy behavior (quickly spreading through bulbils) in its native range as well, especially in disturbed habitats. *O. purpurea* is generally understudied, limiting mitigation and control efforts against this plant as a global weed (Haukka *et al.* 2013). At least five cytotypes have been recorded for this species (J. Suda, unpublished data.), which, given the known relationship between polyploidy and invasiveness (Pandit *et al.* 2011), suggests that ploidy could be contributing to its invasion success. *O. purpurea* further displays substantial variation in many known polyploidy-influenced traits, such as plant size, growth form and degree of selfing/asexual reproduction (K.C. Oberlander, *pers. com*.). In South Africa, the population structure differs between native and invasive populations, with invasive populations often forming dense mats dominated by one morph while native populations consist of a many individuals occurring a few meters apart with equal representation of all three morphs (Manning & Goldblatt 2012). Exploring the correlation between morphology and ploidy in *Oxalis purpurea* could potentially

explain cytotype-driven invasiveness (if present) and population and demographic differences between native and invasive populations.

7. Aims

This study aimed to explore the morphological changes, particularly the Gigas effect, associated with polyploidy in Cape *Oxalis* taxa in general, by comparing trait dimensions between diploids and polyploids. As a first aim, I sought out general trends across the GCFR lineage by comparing select traits for diploids and polyploids of multiple taxa. As a second aim, I set out to study the potential Gigas effect in the weedy species *O. purpurea*. In the latter case I also aimed to explore the relationship between polyploidy, morphological variation and invasiveness between diploid and polyploid *Oxalis purpurea*.

1. The first data chapter aimed to measure the phenotypic consequences of polyploidy at a broad phylogenetic scale, across a range of GCFR *Oxalis* species. Given the well-known consequences of the Gigas effect, I hypothesized that as ploidy increases, so should cell size. Therefore, I expected to observe a substantial and consistent increase in measured traits (stomatal length, epidermal cell size and pollen grain diameter) in polyploids relative to diploids.

2. In the second data chapter, I aimed to determine if there are any correlations between ploidy level, morphological characters and invasiveness within a single *Oxalis* species known to include a diverse polyploid series. It focussed on the morphologically highly variable species *O. purpurea* across its known distribution range, in both weedy and natural populations. I expected to see a substantial and consistent size increase in all measured traits in polyploids. I further expected ploidy level to be correlated to traits that are advantageous in weedy (invasive) populations, such as polyploid-induced increased rates of selfing or asexual reproduction.

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CHAPTER 1

Weak and inconsistent signal of the Gigas effect in *Oxalis* polyploids

Abstract

The Gigas effect is a prominent phenotypic effect of Whole Genome Duplication (WGD). The enlargement of cells and organs (Gigas effect) is thought to confer potential fitness advantages, enabling polyploids to persist and escape minority cytotype exclusion imposed by co-occurring diploids. The Gigas effect is thought to have an effect size of 20-25% larger in polyploids, and this effect size remains consistent across traits. I focused on traits known to be correlated to the Gigas effect at smaller phenotypic scales (stomata length, pollen grain diameter and epidermal cell area), and found *Oxalis* polyploids to deviate from the expected pattern of enlargement. I showed, across 24 species from a wide taxonomic background, that the Gigas effect is extremely small, ranging from 10% in stomata length to 0.1% in epidermal cell area. I also showed that the effect size is highly inconsistent across traits and species, even being reversed in some taxa. The large variation in trait responses may be attributed to local adaptation after WGD and as a result, accessions sampled further apart should display greater variability. By only keeping the geographically closest diploid-polyploid pairs within each species, we showed that geographic distance has no correlation with increased variability in measured traits of polyploids.

1. Introduction

The evolutionary significance of polyploidy and its effect on phenotypic traits are still not well understood. Polyploidy is known to have immediate consequences on the phenotype, especially in plants (Gates 1909, Stebbins 1947), but these effects vary greatly across plant taxa, both in occurrence and magnitude (Smith *et.al.* 1985). Genetic changes brought on by polyploidy, such as changes in non-functional DNA sequence, DNA expression pathways, neofunctionalization and large quantities of DNA allowing greater levels of non-detrimental mutation lead to substantial potential for phenotypic variation in polyploids (Bennet & Leitch 2005, Chen 2007, Leitch & Leitch 2008).

In addition to its morphological effects, polyploidy may also alter physiological processes, such as increasing drought tolerance, increasing photosynthetic activity or even increasing salt tolerance, as well as influencing genetic and epigenetic mechanisms in gene regulation (Levin 2002, te Beest *et.al.* 2012, Ramsey & Ramsey 2014, Wang *et.al.* 2004). These changes may cause changes in ecological interactions of polyploids with other species. Polyploids may, for example, attract a different pollinator than their diploid parents (Levin 1983, Levin 2002). Similarly, increased seed size may affect the distance or effectiveness of the seed dispersal network (Li *et al.* 2004**,** Baack 2005**,** Kreitschitz and Vallès 2007**,** Segraves & Anneberg 2016). Changes in metabolic rates or novel secondary metabolites may even cause changes in their microbial associations (Benitez-Malvido *et.al.*2014, Galen 1999, Policha *et.al.* 2016, Levin 2002, Ramsey & Ramsey 2014).

At the most elemental level the changes induced by polyploidy may simply be attributed to the fact that polyploids have more DNA in each cell. One of the most common phenotypic effects of polyploidy is the Gigas effect – or enlargement of plant traits (Gates 1909, Porturas *et.al.* 2019). With a clear correlation between cell size and WGD (Porturas *et al.* 2019, Balao *et al.* 2011, Wei Na *et al.* 2019, Bennett 1987), increases in the size of plant parts at larger scales have been strongly positively correlated with an increase in ploidy level (Otto & Whitton 2000, Bennet 1971). Such enlargements have been reported at all phenotypic scales, in plant cells, stomata, flowers, leaves and pollen (Altmann *et.al.* 1994, Stebbins 1971, Levin 2002, Knight & Beaulieu 2008). Recent meta-analyses have suggested a substantial effect size for the Gigas effect, at the order of 20-25% for neopolyploids (Porturas *et al.* 2019).

The enlarged cell size associated with the Gigas effect can slow the rate of cell-division in polyploids (Cavalier-Smith, 1978, Bennet 1987, Bennet & Leitch 2005), which in turn can alter their relative growth rate and generation times (Garbutt & Bazzazz 1983, Knight *et al.* 2005, Herben *et.al.* 2012). Having larger cells and organs, and a different growth rate, may give polyploids a competitive advantage relative to their diploid parents by increasing their environmental tolerances and longevity (Stebbins 1971, Levin 2002, Ramsey & Schemske 2002). Wei Na *et.al.* (2019), for example, showed that functional trait divergence due to changes in size and structure contributed strongly to increased fitness of polyploids relative to their diploid parents in the genus *Fragaria* L. Importantly these morphological, physiological and ecological changes displayed in polyploids do not necessarily increase their competitive ability (Wood *et.al.* 2009).

Polyploids can show a variety of epigenetic effects, both advantageous and detrimental (Madlung *et.al.* 2002, Wang *et.al.* 2004). Unexpected phenotypic responses can also arise from developmental trade-offs in polyploids (Prusinkiewicz *et.al.* 2007). For example, cell enlargement could confer an advantage through the production of larger flowers or attraction of a different pollinator, resulting in increased fitness. This will enable the polyploid to escape minority cytotype exclusion when occurring in sympatry with its diploid parents (McIntyre 2012). Larger cells and plant parts can also cause a polyploid to have reduced growth and developmental rates, delaying flowering times (Ramsey & Ramsey 2014, Cavalier-Smith, 1978). This could allow for niche partitioning of a pollinator and lead to the successful establishment of a polyploid population. On the other hand, changes to flower morphology may cause a shift in pollinator host, one that may not always be available. Lower metabolism, as a result of larger cells (meaning metabolites have a longer distance to travel), in polyploids can also enable them to survive conditions where diploids would fail to establish (McIntyre 2012), but then again, slower growth rates could also hamper a polyploid's competitive ability when co-occurring with diploids. In summary, WGD often does affect the phenotype of polyploids and can bring about some overall fitness advantage.

The Greater Cape Floristic Region (GCFR (Bergh *et al.* 2014, Manning and Goldblatt 2012)) of South Africa contains very high levels of endemism and species diversity (Cowling *et al.* 2009). Surprisingly, given that polyploidy has been considered a major driver of species diversification, the GCFR is mostly polyploid poor (Oberlander *et al.* 2016, Rice *et al.* 2019). GCFR members of the genus *Oxalis* (Oxalidaceae) represent a notable exception, with very

high levels of polyploidy recorded among the more than 230 southern African species (Manning and Goldblatt 2012, Krejčíková *et al.* 2013 a, b, c, Snijman 2013, Dreyer *et al.* 2017)). Most *Oxalis* polyploids are believed to be autopolyploids, as the genus shows a low tendency to hybridize successfully (du Preez *et al.* 2018*)*. Given that *Oxalis* is one of few GCFR genera with high rates of WGD, and that it has radiated extensively in the GCFR, it provides an excellent system to test phenotypic effects on polyploids relative to their diploid parents.

It has been shown that, in some polyploids, trait variation may increase with phenotypic scale (Knight & Bealieu 2008). The clearest signals of the Gigas effect may therefore be detectable at the cellular rather than the organ or organismal level, as cellular-level traits may be more exempt from trait plasticity and variation in organ dimensions (Balao *et.al.* 2011, Wei Na *et.al.* 2019). Stomatal guard cells are among the smallest plant cells and rarely undergo endoduplication, rendering them good candidates to measure for the Gigas effect (Melaragno *et al.* 1993). Epidermal cell area and pollen diameter have also been reported as useful traits to measure the Gigas effect (Beaulieu *et al.* 2008**,** Knight & Beaulieu 2008).

This study aimed to measure the potential phenotypic effects of polyploidy using cellular-scale measurements on an array of GCFR *Oxalis* species, specifically the level of expression of the Gigas effect. I measured and compared pollen, stomatal and epidermal cell sizes between diploids and polyploids of the same species across a wide systematic representation of the lineage. If the Gigas effect is operating, or has operated recently, in this lineage, I expect to see a consistent and substantial enlargement in polyploid cells, stomata and pollen relative to diploids.

2. Materials and Methods

Sample Collection:

Samples were collected from the *Oxalis* living research collection at the Stellenbosch University Botanical Garden (SUBG). Ploidy levels of plants in this research collection were previously determined by flow cytometry using protocols and methods as described in Dolezel *et.al.* (2007). Since available polyploids from different species had different cytotypes (ranging from tetraploid (4x) to octaploid (8x) we did not distinguish between ploidy level, only between diploids and polyploids. Additional sampling for *Oxalis livida* Jacq. was done from a nearby

population on Stellenbosch mountain, Stellenbosch, South Africa (-33.947478, 18.879914). For pollen measurements a total of 65 accessions, each from a different population (with 1 to 4 plants per accession, and at least 1 diploid and 1 polyploid accession per species) across 24 species were collected. For epidermal and stomatal measurements, a total of 66 accessions (1 to 4 plants) across 23 species were collected, again including at least one diploid and one polyploidy accession per species. Sample numbers differ due to availability of flowers and anthers of equal height from different morphs.

Pollen diameter:

Oxalis has a tristylous reproductive system with each species including three flower morphs, each with their two anthers whorls and one stigma whorl at three levels in the flower (Barrett 2002). Morphs are distinguished from one another by the position of the stigmas; for example, a Long morph will have the stigmas in the top position and anther whorls at the mid and short positions. Pollen size differs between anther whorls of the flower, with long-positioned anthers usually having the largest pollen and short-positioned anthers the smallest (Ornduff 1972). We controlled for this by collecting pollen from the common anther whorl present in the accessions of each species, as any two plants will always have one common anther whorl position, regardless of morph.

One flower per plant was sampled, and one anther removed from the appropriate whorl. Only one flower was sampled per plant due to limited availability of flowers across individuals and for self-pollination experiments. The entire anthers' contents were gently forced out onto a mounting slide by rolling over the anther with a mounted needle. Pollen was stained with Alexander's stain [10 ml 96% ethanol, 10 mg Malachite green (1 ml of 1% solution in 96% ethanol), 50 ml distilled water, 25 ml glycerol, 5 gm phenol, 5 gm chloral hydrate, 50 mg acid fuchsin (5 ml of 1% solution in water), 5 mg Orange G (0.5 ml of 1% solution in water), glacial acetic acid to the final concentration of 4%] (Alexander 1969). One drop of stain was dripped onto each slide, the stained pollen covered with a cover slip, and slides sealed using nail varnish. Slides were left for a few minutes (up to a maximum of 1 day) before studying them with the aid of a Leica DM500 light microscope (Leica Microsystems, Switzerland). Pictures were taken with an ICC50W mounted camera, LAS EZ software (Leica Microsystems (Switzerland) Ltd, 2016) using the 40X objective lens. Three camera-wide fields (300 x 225 microns) of pollen grains were randomly selected per slide, with 10 pollen grains in each field. For each flower a total of 30 pollen grains were photographed. Pollen grain diameters were

measured using ImageJ software (Schneider *et.al.* 2012). Where possible, pollen grains were measured along their equatorial axis (the largest distance on the pollen grain) to ensure consistency. For wrongly-orientated pollen grains, the largest length was taken as a close approximation of the pollen diameter – these measurements did not differ significantly from one another ($p = 0.1692$) with averages of equatorial pollen measurements = 41.74 µm (Std.dev = 3.583) and other orientations = 42.76 µm (Std. dev =2.928).

Stomatal size:

Stomatal length was measured on sepal stomata rather than leaflet stomata, because leaflet stomata are sunken between massively swollen and protruding epidermal cells in some taxa (Jooste *et al.*, 2016), making measurement difficult. For sepal stomatal measurements, one flower was taken per plant and the three outermost sepals were removed and photographed under a Leica DM500 light microscope (Leica Microsystems, Switzerland) using the 40X objective lens. 10 stomata were measured on the relevant surface (adaxial or abaxial on sepal depending on species) of each of the 3 sepals using ImageJ software (Schneider *et al.* 2012), measuring the parallel length of stomatal guard cells.

Epidermal cell size:

Sepal epidermal cell surface area was measured. Four cells (excluding subsidiary cells) were measured in five different fields of view on three sepals of a single flower per plant. Photos of the five fields of view were taken at 400X magnification on a Leica DM500 light microscope (Leica Microsystems, Switzerland). Photos were kept to the middle of the sepal, starting from the base at the first occurrence of stomata, and moving towards the sepal apex. Cells close to the apex and margins of the sepals were avoided, as these could be much smaller than non-marginal cells. Cell surface area was quantified using the polygon selection function in ImageJ software (Schneider *et al.* 2012).

Statistical analysis:

All analyses were conducted using R software, (R Core Team (2018)), using the lme4 (Bates *et al.* 2014) and afex (Singmann *et al.* 2015) packages and visualised using ggplot2 (Wickham 2016).

I expected measured traits (pollen size, stomatal length and epidermal cell area) to be larger for polyploids showing evidence of the Gigas effect. For pollen diameter a Linear Mixed Model

(random slope and intercept model) was used. Ploidy was set as the fixed factor, with random factors accession nested in species. Due to considerable model violations for both Linear Mixed Models and Generalized Linear Mixed Models, data were log-transformed showing substantial improvement, with minor model assumption violations. Stomata length was modelled using a Linear Mixed Model, with ploidy level as fixed predictor and accession nested in species as random factors. For epidermal cell surface area, data were natural log-transformed after model-fitting on the original data showed severe violations of normality and heteroscedasticity. To account for size differences in response to ploidy level between species, ploidy was modelled as a random slope variable to account for variation in epidermal cell size differences between polyploids of the same species, and species as random factor.

Unexpectedly, I observed a high degree of variation in effect sizes due to ploidy level in all three data sets. This also included diploid-polyploid pairs where the observed pattern was exactly reversed from the pattern expected under the Gigas effect. A possible explanation is a high degree of variability in response variables across species, such that any observed effect of ploidy is masked by other factors. Assuming that accessions from populations nearer to one another were more genetically similar and therefore would have less variability in the response variable, I decided to exclude multiple populations from each species (17 accessions for pollen data and 20 accessions for stomatal and epidermal comparison), keeping only the nearest diploid-polyploid pair for each species. If this assumption was true, one would expect to see a significant increase in the estimated effect size of polyploidy in the smaller data set. Using the same modelling structure, I tested this on a subset of the data consisting only of the closest diploid-polyploid pair for each species.

If across-species variability in pollen/stomatal/epidermal cell size were influencing estimates of ploidy level effects, under the same assumption as before, it would be expected to see greater variability in ploidy effect size estimates as distance between accessions grows. Using effect size coefficient from the models above, I tested the correlation between effect size and distance using Spearmans correlation (Sedgwick 2014).

3. Results

On average, as expected under the Gigas effect, polyploids had larger pollen grains, epidermal cells and stomatal cells than diploids within a given species. The pollen grains of polyploids were on average 1.090 μm larger (p=0.002) than the diploid grains (44 μm), the epidermal cells 1.29 μm^2 larger (p=0.0029) and the stomata 2.59 μm (p=0.0091) larger than conspecific diploids. However, and unexpectedly, there was substantial variation for polyploids in pollen sizes, ranging from -7.1108 μm in *O. callosa* R.Knuth to 16.412 μm in *O. ebracteata* Savign. (*Figure 1.1*). A large part of pollen size variation is due to whorl-specific differences between species, however, the overall response of polyploid populations within a species varied considerably between species, with substantial variation in effect sizes across traits (*Table 1.1*).

Since I included multiple accessions for most species, and I suspected that the effect of polyploidy may be overshadowed by local adaptation of populations that occur geographically far apart, I excluded these additional populations to keep a single diploid-polyploid pair per species. Members of this pair were selected to be the geographically closest populations available. It has to be noted though that one species, *O. livida,* was sampled from a sympatric polyploid-diploid population in Stellenbosch and also yielded variable results across traits.
The exclusion of such additional populations changed very little to the effect size of polypoidy (*Table 1.1).* Polyploids were either much larger or much smaller than diploids. One possible reason for this is that although I excluded some populations (17 accessions for pollen and 20 accessions for stomatal and epidermal comparison for 10 species), many of the included diploid-polyploid pairs were still geographically very far apart (>100 km).

Plotting effect sizes for each species of the reduced dataset to geographic distance between sampled populations (*Figure 1.4*), we found no clear pattern of correlation between geographic distance and polyploid trait variation, pollen (p = 0.1902), epidermal cells (p= 0.4355) and stomata (p = 0.0316) with an average effect size for distance = -0.498.

Table 1.1: Overall effect of polyploidy on pollen size, epidermal cell area and stomatal length. Polyploids, on average, had larger pollen grains ($p < 0.005$), larger epidermal cells ($p < 0.005$) and larger stomata ($p < 0.05$) than diploids, but with very small effect sizes.

Full Data Set				
Measurement	**p-value**	**95% Confidence intervals**	**Effect size of polyploidy**	**Average diploid size**
Pollen size(μm)	0.0020 **	1.035- 1.148	1.090	44.171
Epidermal cell area(μm^2)	0.0029 **	1.111-1.518	1.299	1372.666
Stomatal length(μm)	0.0096 **	0.661-3.819	2.237	25.992
Closest Diploid-Polyploid Pair Data Set				
Measurement	**p-value**	**95% Confidence intervals**	**Effect size of polyploidy**	**Average diploid size**
Pollen size(μm)	0.0023 **	1.035- 1.142	1.087	44.067
Epidermal cell area(μm^2)	0.0007 ***	1.148-1.541	1.330	1345.122
Stomatal length(μm)	0.0008 ***	1.282-4.040	2.661	25.736

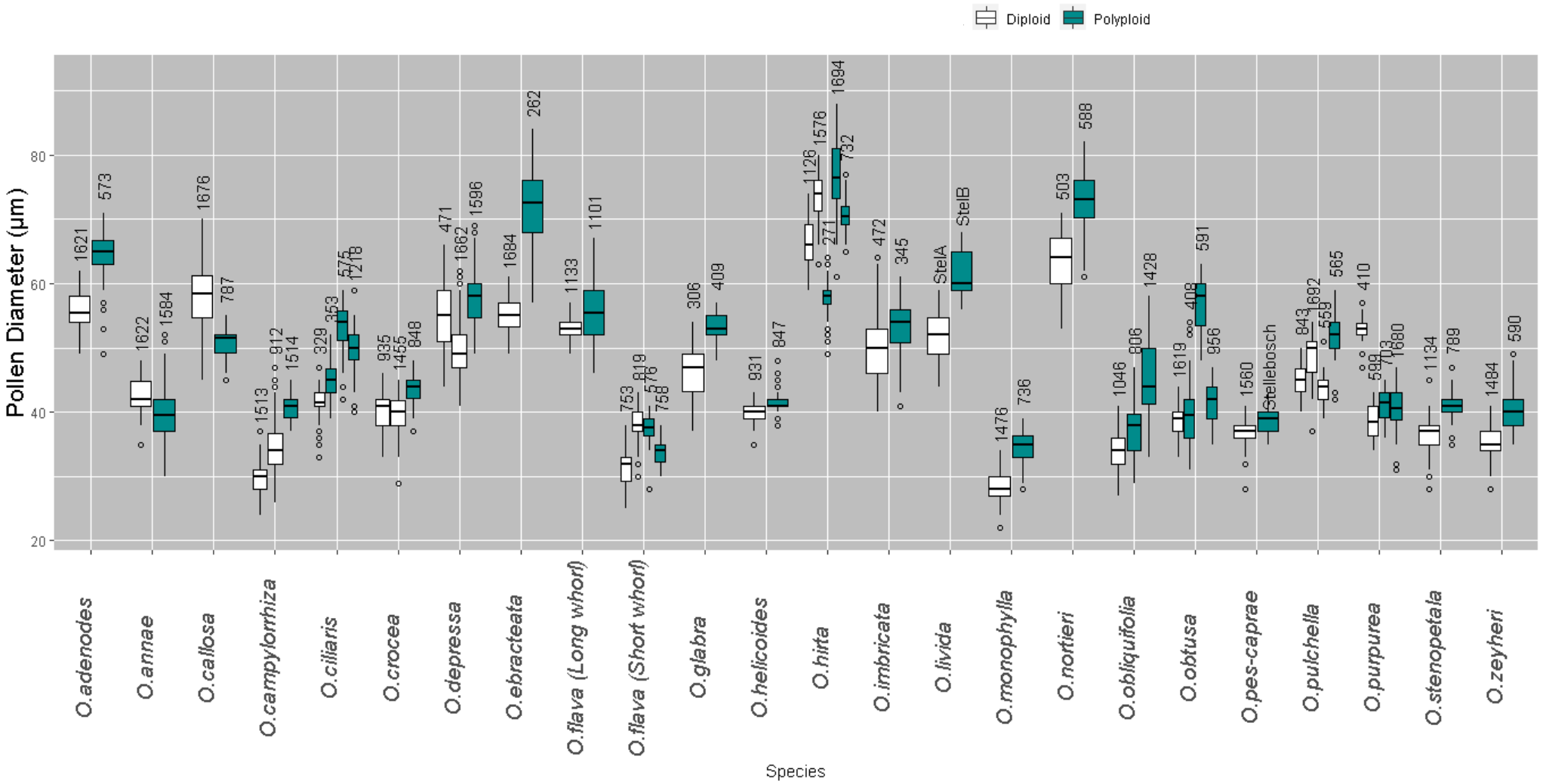

Figure 1.1: Variation in pollen grain sizes of *Oxalis* diploids and polyploids. Diploid-polyploid pairs of the same species were sampled from the same anther whorl height, but anther whorl height differed between species. Box labels refer to accession numbers in the *Oxalis* research collection, SUBG.

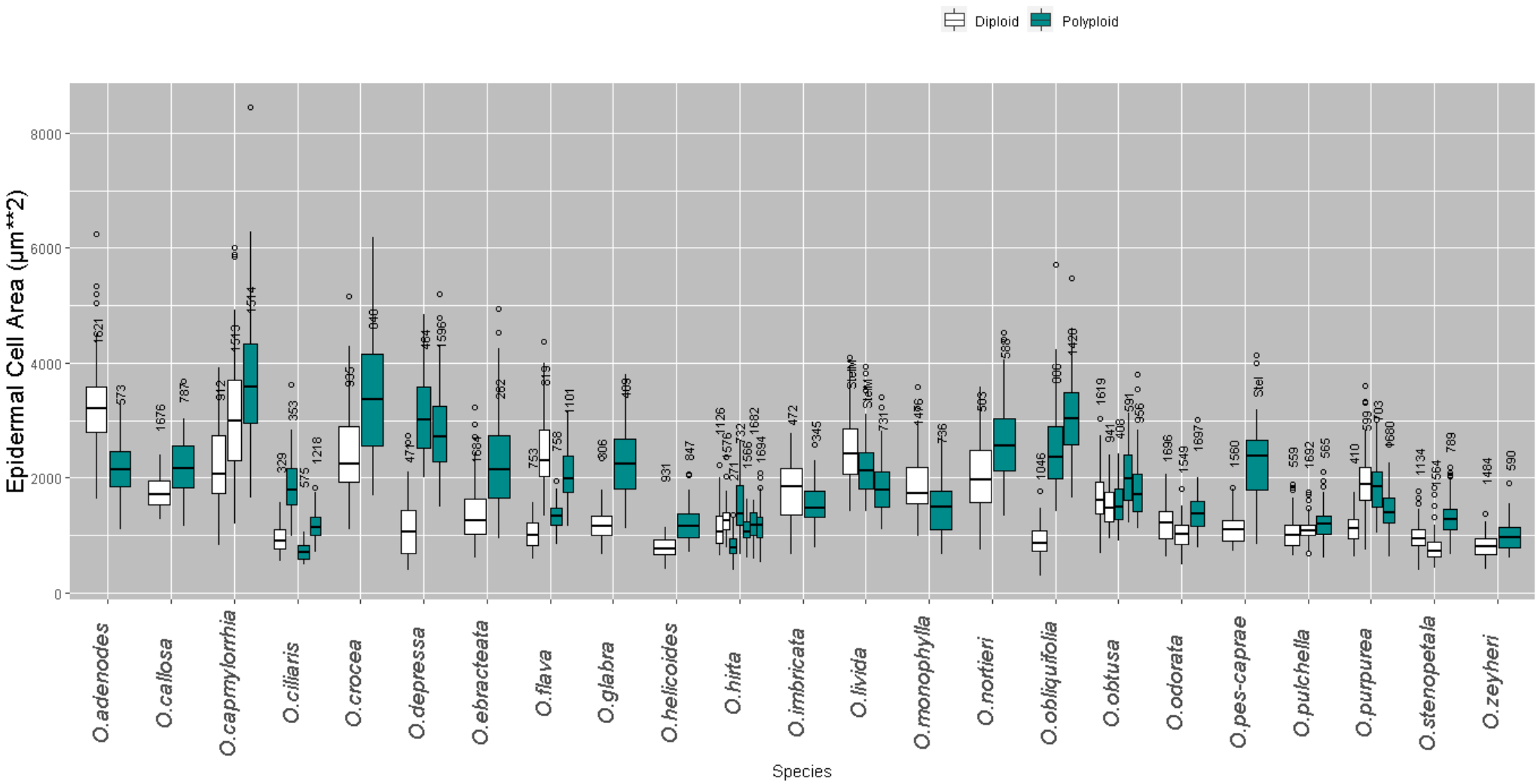

Figure 1.2: Sepal epidermal cell areas of diploid and polyploid individuals of 23 *Oxalis* species. Box labels refer to accession numbers in the living collection of SUBG.

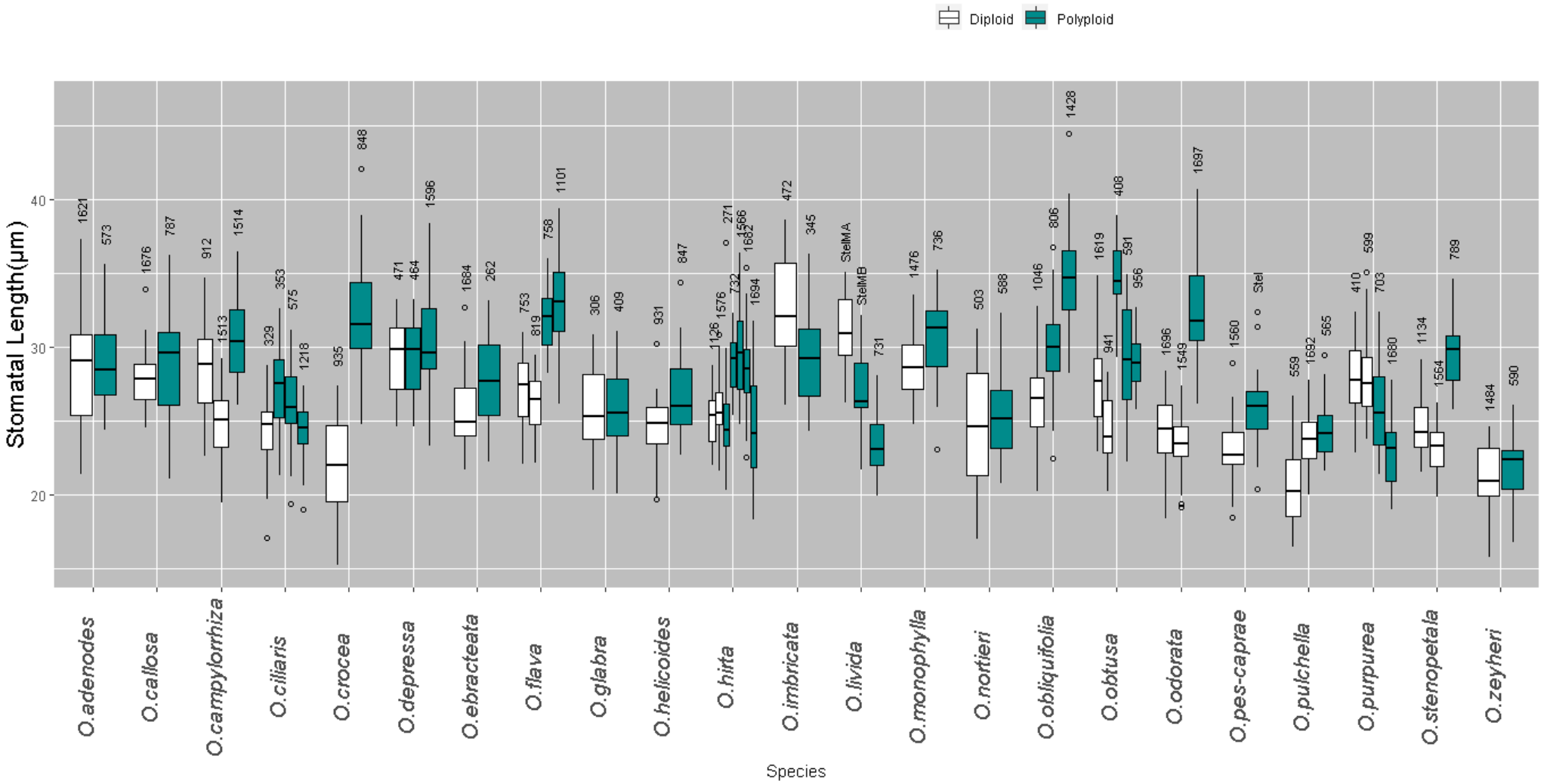

Figure 1.3: Stomatal guard cell lengths of diploid and polyploid accessions of 23 *Oxalis* species. Box labels refers to accession numbers in the living collection at the SUBG.

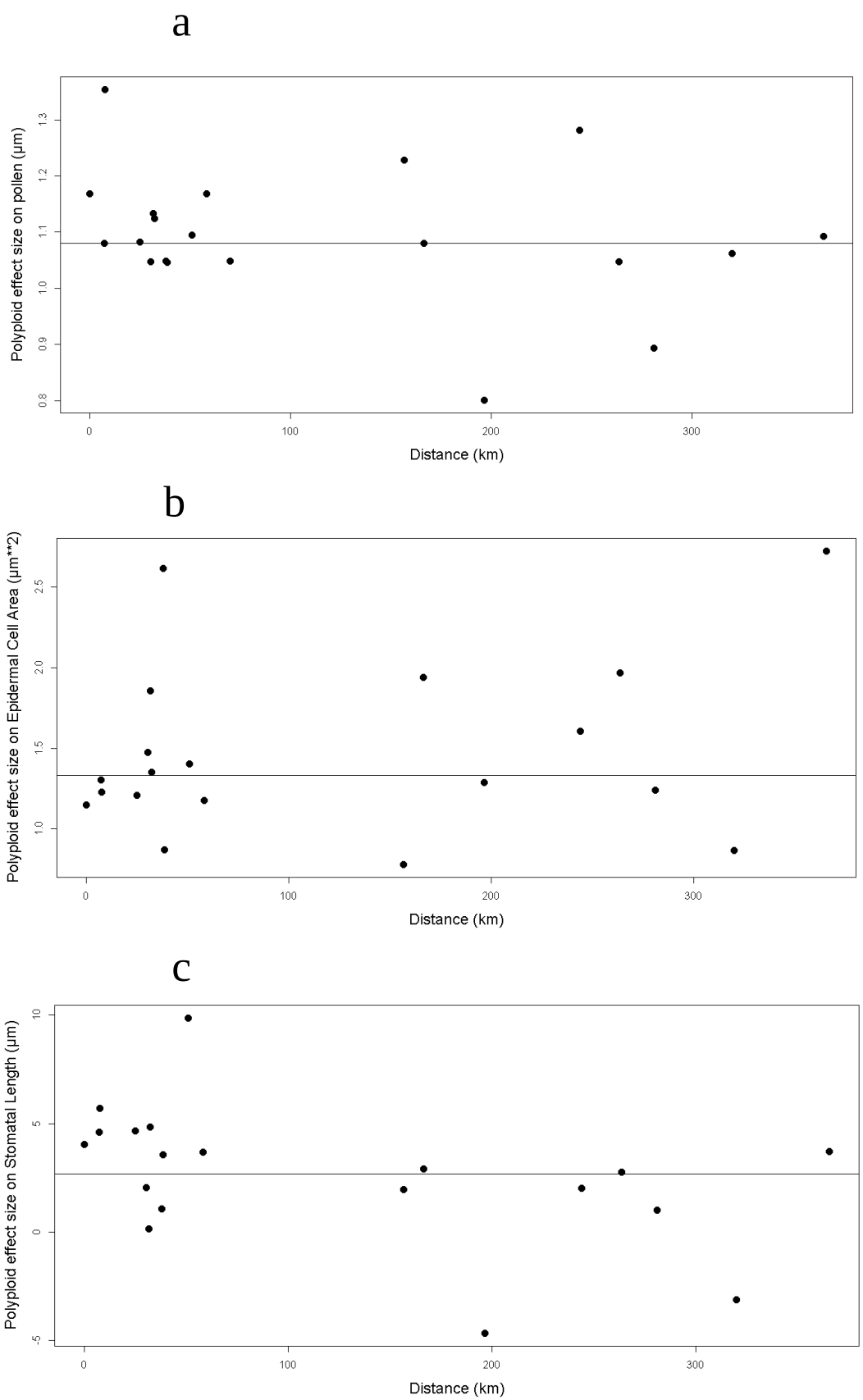

Figure 1.4: **(a)** Effect sizes of polyploidy on pollen diameter mapped against geographic distance between accession collecting sites (p = 0.259). **(b)** Ploidy effect sizes on epidermal cell area mapped against geographic distance between accession collecting sites (p= 0.2802). **(c)** Ploidy effect sizes on stomata length mapped against geographic distance between accession collecting sites (p = 0.049).

4. Discussion & Conclusion

There is some evidence of the Gigas effect in *Oxalis* polyploids as they have on average larger stomata, pollen and epidermal cells ($p<0.05$). Polyploid individuals have pollen grains that are, on average, 1.090 μm larger in diameter than those of diploids, epidermal cells areas that are 1.29 μm^2 larger and stomatal guard cells that are 2.58 μm longer than diploids. These effect sizes are very small, compared to those reported for polyploid angiosperms in general. Porturas et al. (2019) reported estimated effect sizes increases of 20-25% among angiosperms (Porturas *et al.* 2019), while we only found a 2% increase in pollen grain size, 0.1 % increase in epidermal cell area and 10% increase in stomatal length in *Oxalis* polyploids.

Apart from the overall effect sizes, there is considerable variation in sizes across and even within species, and traits. Some polyploids showed the opposite pattern to what was expected under the Gigas effect, while others showed no statistical difference at all. Such a pattern has been documented by various previous authors (Ning *et.al.* 2009, Segraves & Thompson 1999, Trojak-Goluch & Skomra 2013), who found polyploids do not differ significantly from their diploid parents or display considerable variation. The variation in pollen size may be as a result of the unreliability of size differences between reduced and unreduced gametes (Sora *et al.* 2016) which could be reflected across different cytotypes, species and between diploids and polyploids. Ning *et al.* (2009) attributed non-significant changes in polyploid flowers to possible dose effect of the genes involved in *Petunia hybrida* flower development. In another example, *Humulus lupulus* L. polyploids exhibited smaller, shorter roots, shoots, leaves and flowers than diploids, though the authors merely attributed these differences to effects of polyploidization on genomes (Trojak-Goluch & Skomra 2013). It has been shown that higher ploidy levels can adversely affect growth and decrease viability in colchicine-induced polyploids (Viehmannová *et al.* 2009). It is, however, currently thought that natural polyploids that display these exceptions to the Gigas effect are in the minority and that, on average, polyploids do trend towards larger cells, gametes and stomata with a consistent effect size across traits (Porturas *et.al.* 2019). My results contradict this view and suggest that polyploids not displaying the classical Gigas response might be more common in *Oxalis*.

Tetraploid *Heuchera grossulariifolia* Rydb. plants showed the predicted increase in size as is expected under the Gigas effect, but with large variation in flower morphology and flowering

times (Segraves & Thompson 1999). According to the authors, this variation may be a result of recurrent polyploid formation and/or natural selection due to the wide geographic range from which this species was sampled. Interestingly, the effect size of polyploidy in most previously recorded polyploids remained constant across the entire plant (Porturas *et.al.* 2019). Again, our results did not fit this pattern, as I found no constant effect size for polyploidy across measurements of stomatal length, epidermal cell area and pollen diameter. Some polyploids have larger pollen relative to their diploids, but smaller stomata and epidermal cells (*Figure 1.1, 1.2, 1.3*). Overall polyploids of different accessions seem to vary considerably relative to their diploid counterparts.

It has been suggested that polyploids would have an increase in variation of trait responses and sizes, although Porturas *et.al.* (2019) showed that this is not the case in general. Even though I found large variation in trait sizes, this was true for both diploids and polyploids (*Figure 1.4).* To test the idea of increased variation as a result of polyploidy from the sampled data, I excluded additional polyploid accessions from diploid-polyploid pairs with multiple accessions to leave the closest diploid-polyploid pair. I argued that if the original size increase due to the Gigas has been masked by subsequent local adaptation, that nearest neighbor diploid-polyploid populations (using geographical distance as a course proxy for genetic distance) would have larger and more consistent effect sizes. I, however, did not find a significant or strong correlation between geographic distance and increased variation. For both epidermal cell area and pollen grain diameter, the correlation between increased variation and geographic distance was non-significant. Stomata length was marginally different between diploid and polyploid individuals ($p = 0.0316$) with a very small effect size (-0.498), suggesting that variation in trait sizes does not increase as a result of polyploidy, or that ploidy plays a very small role in the observed variation.

These results highlighted two potential shortcomings of this study. Firstly, given the highly heterogeneous geographic landscape of the Cape, with massive variation in microclimates (Linder, 2003), geographic distance might not be the most accurate predictor of variable trait response due to local adaptation, and a more direct measure of relatedness might show stronger patterns. Secondly, within-species sampling from different polyploid and diploid populations was very small, using accessions instead of extensive within-population sampling. This may have hampered our ability to observe within population variation.

Having accounted for the possible variation between species and for the unexpected variation between populations within species, I do not find any evidence to suggest that size differences due to the Gigas effect are a reliable way to distinguish between Cape *Oxalis* diploids and polyploids. This agrees with results of Knight *et.al.* (2010), who concluded, using 464 species across gymnosperms and angiosperms in a regression analysis, that pollen size was not a reliable measure to distinguish between ploidy levels. Furthermore, I found little evidence to suggest that polyploidy-driven size variation is the major cause of or even a contributor to the massive morphological variation observed in this genus.

Alternative reasons for masking of the Gigas, at least for the pollen data in *Oxalis*, could include natural pollen size variability. Pollen of tristylous species decreases in size from long level anthers through to short level anthers (Barrett 1993), and given that tristyly is an unstable system, pollen size variations may vary between species with variation on the maintenance of a tristylous system (Ornduff 1972). I accommodated for the known differences of tristylous pollen by sampling from similar anther whorls for within species pairings of a diploid and polyploid. Given this known effect of tristyly on pollen size, we expected variation in pollen dimensions within a species, but the magnitude of pollen size variation between species was not expected. In a single whorl, *Oxalis* species may cover more than half the size range for pollen grains as recorded for the entire angiosperm lineage (Knight *et.al.* 2010). It has been suggested that the length of the style may influence the size of pollen grains (Cruden 2009), but it may be that underlying genetic mechanisms may cause both longer styles and larger pollen grains. Another possible explanation for the observed massive variation in pollen size may be a high prevalence of cytomixis. Cytomixis (the migration of the nucleus from one plant cell to another) has been shown to influence pollen grain size and viability (Singhal & Kumar 2008). A last possible explanation for the great variability in observed pollen diameter may be the nutrient heterogeneity of the Cape soils. Soil nitrogen was found to affect average pollen size and viability in *Cucurbita pepo* L. (Lau & Stephenson 1993). As none of these potential explanations have been tested in *Oxalis*, they all remain hypotheses that may help explain the large variation in pollen size observed in this genus.

The large variation in *Oxalis* polyploid traits may also reflect multiple and recurrent formations of *Oxalis* polyploids. Becker *et al.* (2020; Chapter 2) conducted extensive sampling of *O. purpurea* populations and cytotype distribution. They observed a near-continuum of relative genome sizes between putative tetra- penta- and hexaploids which is consistent with multiple

and recurrent formation of *Oxalis* polyploids. The age of WGD events in the different sampled *Oxalis* species is unknown and almost certainly varies. I therefore argue that post-WGD local adaptation in heterogeneous Cape environments may have diluted the initial effects of cell enlargement in *Oxalis* neopolyploids to varying degrees. The fact that these polyploids may differ in age between, and even within sampled species (if multiple WGD events), could have further increased trait variation, resulting in the variation detected in this study. Therefore, to understand the patterns of variation in polyploid traits, the inconsistency across traits and the small effect size of the Gigas effect detected among *Oxalis* polyploids, future studies should focus on determining the age of WGD events in different *Oxalis* species, It would also be very interesting to observe phenotypic changes in synthesized neopolyploids, and to compare them to the results of this study.

In conclusion, I find little and inconsistent evidence for a strong Gigas effect across the GCFR *Oxalis* clade, despite this lineage being a good candidate for this effect. Newly formed *Oxalis* polyploids may show a temporary Gigas effect immediately after formation, but if so, this distinctive increase in size traits seems to dissolve in the massive natural trait variation observed in this genus. To conclusively say that polyploids show no distinctive difference to diploids, deeper sampling of within species populations is needed to account for the considerable natural variation we detected. Based on the small sample sizes used here, I can only conclude that Cape *Oxalis* polyploids show no increase in size of pollen and cellular measurements relative to their diploid parents.

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CHAPTER 2

Whole Genome Duplication facilitates invasiveness but not through the Gigas effect in *Oxalis purpurea* polyploids

Abstract

The Gigas effect (enlargement of plant traits) brought on by Whole Genome Duplication (WGD) often causes diverse physiological and ecological effects. These changes may enable a polyploid to successfully establish in a novel environment. As a result, WGD has been linked with increased invasiveness of plants via traits such as increased selfing or clonality. By doing extensive sampling of 20 populations of *Oxalis purpurea*, a known global and locally invasive weedy species, I tested whether *O. purpurea* polyploids exhibit the Gigas effect and/or increase in invasive traits. I used previously known correlates of the Gigas effect at smaller phenotypic scales (stomatal length, epidermal cell area and pollen grain diameter) and showed that *O. purpurea* polyploids display a significant but very weak and inconsistently expressed Gigas effect. In addition, I measured 12 floral and leaf traits, and showed that as phenotypic scale increases, most evidence for the Gigas disappears. All tested diploid and polyploid *Oxalis purpurea* plants were strongly self-incompatible. In contrast, measures of clonality revealed that polyploid *O. purpurea* plants produce significantly more and heavier bulbils than diploids. This suggests that size increase due to WGD plays an ephemeral role in promoting invasiveness in *O. purpurea*, and that the Gigas effect may be unevenly expressed, with polyploids storing more resources underground than above-ground.

1. Introduction

Polyploidy, or whole genome duplication (WGD), is regarded as a major evolutionary force in plants (Mable 2003, Gregory & Mable 2005). Polyploidy is often associated with large radiations - for example, the angiosperm genome shows evidence of multiple episodic events of WGD (Jiao *et al.* 2011,2012, Cui *et al.* 2006, Soltis *et al.* 2009, Van de Peer *et al.* 2009, Van de Peer 2011). Furthermore, polyploidy can cause instant speciation and is often linked with increased speciation and diversification in lineages (Levin 1983, 2002). But the long-term evolutionary significance of WGD is still an ongoing debate, with some evidence suggesting that WGD acts as evolutionary noise, being produced by evolutionary processes, but not actively contributing to lineage divergence and persistence (Otto & Whitton 2000, Ning *et al.* 2009, Stebbins 1950, 1971).

Theoretical predictions state that polyploids should rarely be able to successfully establish in nature (Levin 1983, Fowler and Levin 1984, 2016, Felber 1991, Baack 2005). The main reason for this is that polyploids are subjected to minority cytotype exclusion (Levin 1975), a principle suggesting polyploid cytotypes (cytotype referring to the number of chromosome sets) are subjected to lower rates of outcrossing and fertilization with cytotypes of the same level, in a diploid dominated population. Furthermore, larger genomes might be under severe environmental constraints, limiting the distribution of polyploids to less extreme environments (Knight *et al.* 2005, Carta & Peruzzi 2016). Lastly, polyploids also diversify at lower rates than diploids (Mayrose *et al.* 2011) raising the question, why do we find polyploids at such high frequencies and abundance in nature?

The evolutionary significance of polyploids cannot be appreciated without an understanding of how they establish and persist. It has been shown that WGD brings about phenotypic changes and generates ecological diversity (Thompson *et al.* 2004, Thompson & Merg 2008, Oswald & Nuismer 2007, 2011, Aravanitis *et al.* 2010, Boalt *et al.* 2010, Ramsey 2011, Martin & Husband 2013, Strong & Ayres 2013, Ramsey & Ramsey 2014, Segraves & Thompson 1999). After formation, they may also show immediate niche differentiation relative to diploids (Ramsey 2011, Levin 2011, Soltis 1984, Theodoridis *et al.* 2013) and by doing so they can escape minority cytotype exclusion by forming an isolated, similar-cytotype population.

For a diversity of reasons, polyploids may enjoy fitness benefits relative to their diploid parents.

Firstly, polyploids may undergo various genetic changes (Anssour *et al.* 2009) such as changes to genetic structure (Lim *et al.* 2008, Chester *et al.* 2012), gene loss or modification (Wang & Paterson 2011, Kashkush *et al.* 2002), altered gene expression (Ainouche *et al.* 2012,Hegarty *et al.* 2005) and the formation of new gene functions or the division of functions in a gene (Ohno 1970, Lynch & Conery 2000, Lynch & Force 2000, Prince & Pickett 2002, Adams & Wendel 2004, 2005).

Secondly, polyploids may experience a breakdown of genetic self-incompatibility systems (Richards 1997, Xiong *et al.* 2011). Through this mechanism, polyploids may also escape minority cytotype exclusion by having a higher selfing rate – enabling polyploids to increase their numbers and reduce the exclusion imposed by the dominance of diploid cytotypes. Such changes could therefore enhance fitness, given that reproductive isolation can be further enforced in polyploids through geographic separation, flowering asynchrony, pollinator fidelity, self-pollination, gametic selection and postzygotic isolation (Husband & Sabara 2004).

The third major factor that may provide polyploids a fitness advantage over diploids is the Gigas effect. This Gigas effect refers to an enlargement of polyploid morphological traits and is the most common phenotypic change associated with polyploidy. It was named after the plant species *Oenothera lamarckiana* mut. *Gigas*, the species in which this phenomenon was first described (Gates 1909). It is thought that as the amount of DNA in a cell increases, so does the size of the cell. This, in turn, results in enlargement of the polyploid plant body, gametes and floral structures (Lutz 1907, Porturas *et al.* 2019). Although such enlargement can be beneficial in and of its own, by being physically larger a polyploid can outcompete diploids for space and sunlight, this increase in size at the cellular, organ and/or organismal level can further lead to diverse physiological changes, including slower growth rates. This may explain why some polyploids do better in novel niches or show signs of flowering asynchrony (Levin 1983, 2002, Warner & Edwards 1993, Coate *et al.* 2012, 2013, Wang *et al.* 2013, Ramsey 2011).

As a result, the Gigas effect, selfing rate and niche differentiation have become useful characters to distinguish between polyploids and their diploid parents (Trojak-Goluch & Skomra 2013, Baker *et al.* 2017, Lavania 2020), without the need to use expensive and/or time-consuming methods and equipment. Given these changes, polyploids may show a higher propensity to invade. Characters enabling polyploids to escape minority cytotype exclusion, either through increased selfing rates or changes to physiological and ecological traits as a

result of the Gigas effect, may be viewed as pre-adapted traits that enable polyploids to increase propagule pressure in introduced environments (Pandit *et al.* 2006, 2011).

Although commonly associated with polyploidy, the universality and reliability of the above-mentioned characters for distinguishing polyploids from diploids varies, with many studies showing mixed results in niche occupation and successful establishment (Martin & Husband 2009, Theodoridis *et al.* 2013, Glennon *et al.* 2014, Harbert *et al.* 2014, Soltis *et al.* 2015). Exceptions to the Gigas effect have also been recorded, where no difference between cytotypes could be detected, or even instances where polyploids were found to have smaller cells than diploids (Trojak-Goluch & Skomra 2013). Becker *et al.* (2020; Chapter 1) assessed the expression of the Gigas effect among Cape members of the genus *Oxalis*, and found polyploids to be significantly different to diploids (on average), but that the expected consistency of the Gigas effect across polyploids, and across measured traits (e.g., Porturas *et al.* 2019) was absent. Becker *et al.* (2020; Chapter 1) found substantial variation in the relationship between pollen size, stomatal length, epidermal cell area and ploidy level across various species. They further recorded highly inconsistent patterns between traits, with some polyploids showing smaller pollen, but larger stomata, than conspecific diploids, while others showed the reverse.

Oxalis has more than 230 species currently recognized in southern Africa (Manning and Goldblatt, 2012). Many of these species are extremely morphologically variable and have been lumped together as species complexes (Salter 1944). Interestingly, many of these species complexes are also associated with extensive polyploid series. *Oxalis obtusa* Jacq., regarded as one of the most widespread and variable species in South African *Oxalis* (Salter, 1944) has seven recorded cytotypes. Krejčíková *et al.* (2013 a, b) found some correlation between the different cytotypes of this species and environmental parameters such as vegetation type and precipitation. To date no other studies have explored the possibility of a link between morphological variation and ploidy level in any of the other *Oxalis* species complexes.

Oxalis purpurea L. represents another very variable group species with an extensive geographical range and is known to include at least five different cytotypes (H. Suda, unpublished data). It is a well-known invasive weed both in its native range and in Australia (Rozefelds *et al.* 1999, Cuevas *et al.* 2004, Paynter *et al.* 1968), the Mediterranean basin, Algeria and California (Randall 2012, Sanz Elorza *et al.* 2004, Randall 2007). It also displays weedy behavior in its native range, South Africa, especially in disturbed habitats.

The native biology of this species remains understudied, which hampers efforts to control it in its invaded range (Haukka *et al.* 2013). Since WGD is often linked with increased selfing rates and clonality (van Drunen & Husband 2019), and polyploids may experience advantages due to the Gigas effect, ploidy may play a role in promoting invasions of this species. If WGD in this species could be linked to morphological and/or physiological traits of *O. purpurea*, we may gain insight into why this species is such a notorious invader, or even which cytotypes are the most invasive. *O. purpurea* thus provides a good model system to test for distinct differences between polyploids and diploids given its widespread distribution range, common occurrence, variable morphology and large cytotype variation.

The aim of this study was to use *O. purpurea* for extensive sampling of known correlates with ploidy level (*i.e.* pollen grain diameter, epidermal cell area and stomata length). This may alleviate some of the problems experienced by Becker *et al.* (2020; Chapter 1), to tease out WGD-related signal from the considerable degree of observed trait variation with small sample sizes per species. Furthermore, I aimed to measure leaf and flower traits to assess the extent and predictability of the Gigas effect in *O. purpurea*. In doing so, I hoped to detect distinct phenotypic characters that separate diploids from polyploids in this species. I hypothesized that polyploids would show the predicted pattern of substantial and consistent cell and trait enlargement. Lastly, to test the potential role of WGD in invasiveness, I assessed levels of self-pollination and underground clonal propagule (i.e. bulbil) production across ploidy levels. I hypothesized that polyploids would show higher levels of self-pollination and clonality relative to their diploid progenitors.

2. Materials and Methods

Sample Collection:

O. purpurea was extensively sampled across its entire range in the Western Cape during 2019 (*Figure 2.1*). The bulbs of 12 plants, four per morph (Morphs are defined by the position of the stigma relative to two anther whorls, in a tristylous reproductive system there are three morphs), were dug up in 20 populations, and samples were taken one or more meters apart to minimize the sampling of clones. Plants were planted in plastic bags containing a 50:50 mixture of sand and potting soil mix, filled to a depth of 10 cm. The bulbs were positioned at a depth of 2.5 cm below the surface, and plants were left to acclimate and go dormant naturally. Plants were watered twice a week, for 5 weeks until the end of September when they typically go

fully dormant in nature. At plant emergence in March 2020, all bagged plants were moved to a location with full mid-day sun in a common garden experiment setup. Bags were moved and rotated on a weekly basis to prevent possible micro-climate effects on individual plants.

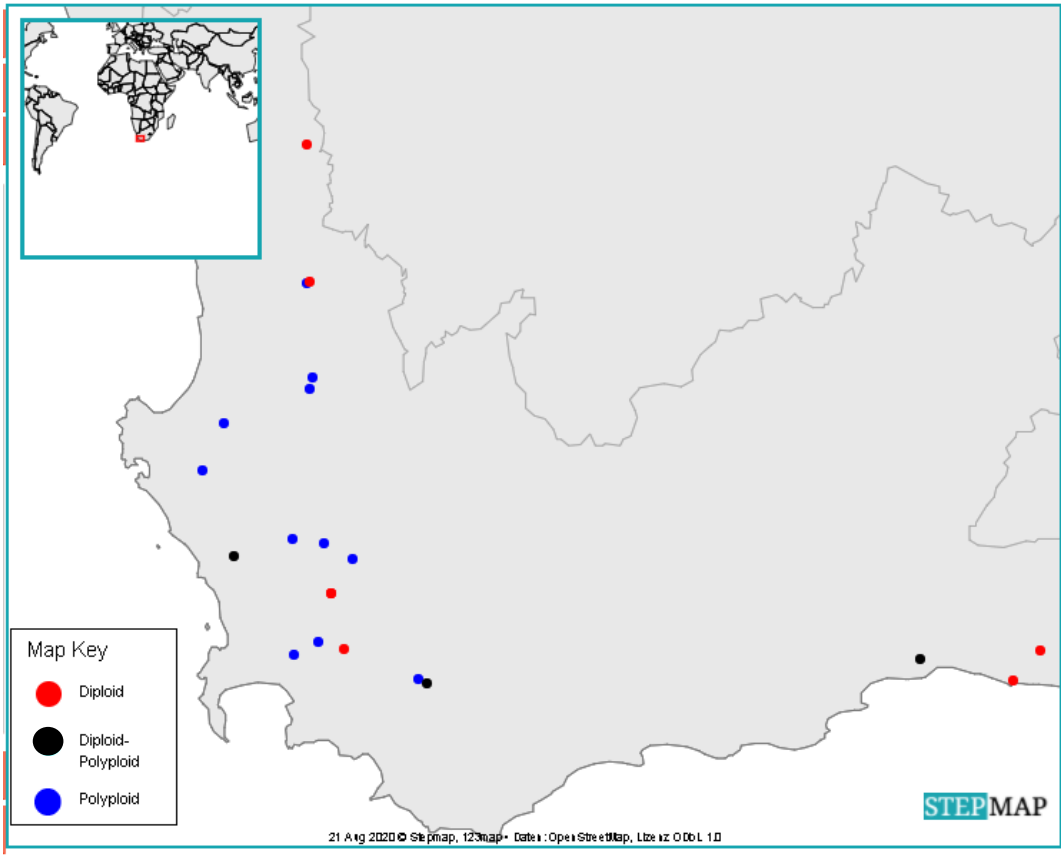

Figure 2.1: Map of *Oxalis purpurea* sampling localities and cytotype distributions across the Western Cape Province, South Africa. Twelve plants were sampled from each site, with four plants per morph.

Flow cytometry:

The ploidy level of all sampled plants was determined via flow-cytometry using the DAPI stain (Dolezel *et al.* 2007). Leaf material was sampled for all plants in the common garden setup, silica dried and used to determine relative genome size, which mostly allowed easy assignment to diploid or polyploid cytotypes. Although I did not confirm ploidy status with chromosome squashes, relative diploid genome size estimates agreed with confirmed diploids of this species using the same method and standard (H. Suda, unpublished data). The following method was followed:

Two-step procedure using Otto I+II buffers

1. Mix fluorochrome (DAPI, 4 µg/ml) and Otto II buffer (2 µl/ml. (DAPI-staining: 25 ml Otto II buffer + 1 ml DAPI stock solution + 50 µl ß-mercaptoethanol)

2. Remove silica dried sample (typically 1 cm^2) and cut with a new razor blade, along with test-standard (1cm^2 - *Glycine max* cv. Polanka, 2.50, 2C-DNA content), in a petri dish containing 1 ml of ice-cold Otto I buffer
3. Filter the suspension through a 42 µm nylon mesh into a cuvette.
4. Add 1 ml of Otto II buffer supplemented with fluorochrome to filtered samples, shake well.
5. Analyse relative DNA content of isolated nuclei

Because relative genome size could not often distinguish between suspected cytotypes (*Figure 2.2*) at higher ploidy levels (tetraploid, pentaploid, hexaploidy), and we could not confirm these cytotypes using chromosome squash techniques, for analysis we lumped all triploids and higher into a single polyploid category.

Pollen diameter:

In order to assess the full extent of pollen variation of this tristylous species, I sampled from both anther levels of all three morphs to examine anther level-specific variation between plants and populations.

One flower was sampled per plant, and one anther was removed from each anther whorl. Only one flower was sampled per plant due to limited availability of flowers across individuals and for self-pollination experiments. The entire anthers' contents were gently forced out onto a mounting slide by rolling over the anther with a mounted needle. Pollen was the stained for viability using Alexander stain [10 ml 96% ethanol, 10 mg Malachite green (1 ml of 1% solution in 96% ethanol), 50 ml distilled water, 25 ml glycerol, 5 gm phenol, 5 gm chloral hydrate, 50 mg acid fuchsin (5 ml of 1% solution in water), 5 mg Orange G (0.5 ml of 1% solution in water), Glacial Acetic acid to the final concentration of 4%] (Alexander 1969).

After staining, the stained pollen grains were covered with a cover slip, and the microscope slides were sealed using nail varnish. Slides were left for a few minutes (up to a maximum of 1 day) before studying them with the aid of a Leica DM500 light microscope (Leica Microsystems, Switzerland). Pictures were taken with an ICC50W mounted camera, LAS EZ software (Leica Microsystems (Switzerland) Ltd, 2016) using the 40X objective lens. Three camera-wide fields (300 x 225 microns) of pollen grains were randomly chosen per slide, with 10 pollen grains in each field. A total of 60 pollen grains were photographed per flower, 30

from each anther whorl. Pollen grain diameter was later measured using ImageJ software (Schneider *et al.* 2012). As far as possible, pollen grains were measured along their equatorial axis (the longest axis of an *Oxalis* pollen grain) to ensure consistency. For wrongly-orientated pollen grains, the largest length was taken as a close approximation of the pollen diameter – these measurements did not differ significantly from one another ($p = 0.1692$) with averages of equatorial pollen measurements = 41.74 µm (Std. dev = 3.583) and other orientations = 42.76 µm (Std. dev =2.928).

Stomatal size:

The stomatal length of adaxial sepal stomata were measured instead of leaflet stomata, because this would make these measurements directly comparable to those of Becker *et al.* (2020; Chapter 1). Jooste *et al.* 2016 provided support that stomatal density decreased as stomatal size increased. Due to time constraints I did not include stomatal density given its known relationship to stomatal size. The three outermost sepals were removed from one flower per plant and photographed using a Leica DM500 light microscope (Leica Microsystems, Switzerland) at 40X magnification. Ten stomata were measured on the adaxial surface of each of the 3 sepals using ImageJ software (Schneider *et al.* 2012), measuring the parallel length of stomatal guard cells.

Epidermal cell size:

Epidermal cells were also measured on the sepals for the same reasons stated above. Four randomly chosen cells (excluding subsidiary cells) were measured in five fields of view on three sepals of a single flower per plant. Photos were taken at 40X magnification on a Leica DM500 light microscope (Leica Microsystems, Switzerland). Photos were taken in the middle part of each sepal, starting from the base at the first occurrence of stomata, and moving towards the sepal apex. Measurements were made using the polygon selection function in ImageJ software (Schneider *et al.* 2012) to quantify cell surface area.

Flower and leaf morphometrics

A total of twelve plant traits was measured using digital calipers accurate to 0.01 mm. The following leaf traits were included: leaflet length, leaflet width, leaflet thickness, petiole length. The following flower traits were included: petal length, petal width, sepal length, sepal width, bract length, bract width, peduncle length, peduncle-to-bract-length. Using plants from the common garden experiment, one flower and one leaf were used per plant. I only used one leaf

as a result of inconsistent flowering and limited flowers available. I consistently sampled the largest, oldest leaf from each plant. Due to considerable mortality and failure to flower, we ended up sampling 151 plants for leaf traits and 112 plants for flower traits.

Self-pollination and clonality

Self-pollination experiments were done using mid-morph flowers only in order to limit potential statistical complications associated with inter-morph variation between plants. Flowers were emasculated after self-pollination since anthers were required to provide pollen from the same plant. Using the plants in the common garden experiment, 20 mid morph flowers were self-pollinated using pollen from the long whorl of the same flower. After pollination, petals were removed, and plants were kept in a partially enclosed area. All newly formed flowers were also removed until such time as the capsules were fully formed or the seeds aborted. Seed set was taken as a measure of successful self-pollination.

I assessed the increase in below-ground bulb (i.e. propagule biomass) and number of bulbils produced per plant as potential estimators of invasiveness. In nature, *Oxalis purpurea* emerges in March before the onset of winter rains and grows above-ground for six to seven months up to about September/October, when plants return to dormancy below ground. After going dormant in 2019, all bulbs were dug up and bulbils removed, with the primary bulb weighed (wet mass) before being re-planted. The bulbs were dug up after the plants went dormant in December 2020. Bulb and bulbils were weighed together (wet mass) to estimate the amount of additional propagule biomass a plant managed to produce during one growing season. The remains of the contractile root, lateral roots and rhizome were removed before measurement. Bulbil production during the one active growth season was quantified by counting the number of bulbils formed between 2019 and December 2020.

Statistical analysis:

All analyses were conducted using R software, (R Core Team (2018)), using the lme4 (Bates *et al.* 2014) and afex (Singmann *et al.* 2015) packages and visualized using ggplot2 (Wickham 2016).

For pollen diameter, a Generalized Linear Mixed Model was used with a gamma family distribution and log link function as these generated the smallest violations in qqplots and variance plots after model fitting. Ploidy and anther whorl were set as fixed factors and

Individual nested in Population were set as random variables. Transformations of the original response variable did not yield better model fits.

Stomatal length was modelled using a linear mixed model with ploidy as fixed factor and individual within Population as random factor. Epidermal cell area was also modelled using a linear mixed model, but due to normality and some heteroscedasticity violations, data were log-transformed showing strong improvement in both.

I used Principle Component Analyses (PCA) to identify leaf and floral traits that potentially covaried with ploidy level. Afterwards these were modelled using a linear and generalized linear mixed model with family "inverse gaussian" and the appropriate link function. Due to strong model assumption violations, petal length, peduncle length, peduncle-to-bract and leaflet width were log-transformed with considerable improvement and modelled in a linear mixed model context. All morphometric trait p-values were corrected for multiple testing using the Benjamini–Hochberg correction (Thissen *et al.* 2002).

Bulbil count was modelled using a generalized linear mixed model with a poisson distribution (link = square root) with ploidy as fixed factor and population as a random factor, while bulb growth (total mass in 2020 – mass in 2019) was modelled using a linear mixed model with ploidy as fixed factor and population as random factor. Since selfing experiments yielded no seeds for any of the self-pollinations, no analysis was needed.

3. Results

Cytotype determinations for 240 plants revealed seven mixed-ploidy polyploid populations (consisting of tetraploid-pentaploid, tetraploid-hexaploid and pentaploid-hexaploid mixes) and three diploid-polyploid populations (only containing either one diploid or one polyploid in sympatry for sampled plants). Of the remaining populations, seven were diploid and four were single-cytotype polyploid populations. For higher cytotypes (*i.e* .tetra-, penta- and hexaploids) relative genome size showed strong overlap, making absolute distinction between cytotypes difficult.

Traits at smaller phenotypic scales showed strong significance with polyploids generally having larger pollen ($p = 0.0242$), stomata ($p < 0.005$) and epidermal cells ($p < 0.005$). The

effect size of polyploidy was, however, substantially smaller than expected for these traits (summarized in *Table 2.1*). Pollen size also differed significantly between anther whorls of different lengths in this tristylous plant.

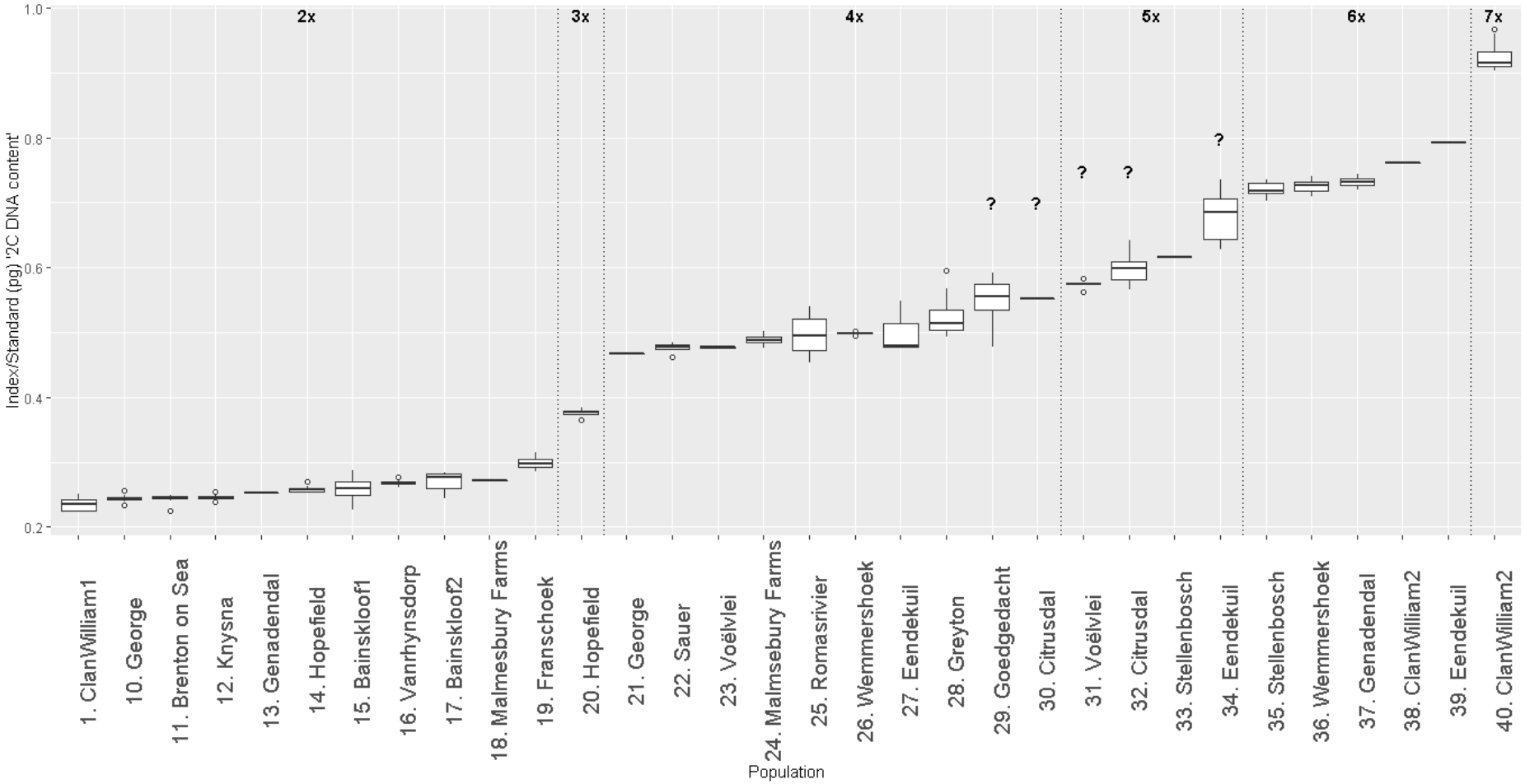

Figure 2.2: Relative genome size against ploidy level for *Oxalis purpurea* populations showing cytotype distribution per population. Note the obscure boundaries between tetraploids, pentaploids and hexaploids.

PCA showed negative correlations between ploidy level and petal length, bract length and petal width among flower traits and leaflet thickness for leaf traits (*Figure 2.3*)

Figure 2.3: Biplots of leaf (a) and flower (b) traits and associated weights on PC1 and PC2. Potential predictors were modelled in a Generalized Linear Mixed Model context to test for a significant relationship between ploidy and the trait. For flower traits PC1 captured 41% and PC2 22% of the total variance. For leaf traits, PC1 captured 65% and PC2 31% of the total variance.

Morphometric measurements varied greatly in significance and effect sizes for traits at a higher phenotypic scale, with only petal width ($p < 0.005$) and leaflet thickness ($p = 0.015$) showing significance after correcting for multiple tests (*Table 2.1*).

Finally, when we tested the ecological implications of WGD, we found a significantly bigger increase in bulb mass among polyploids than among diploids ($p = 0.045$) (*Figure 2.6*). On average, polyploid bulbs increased in mass twice as much as diploids (*Table 2.1*). Polyploids also produced significantly more clonal bulbils than diploids ($p<0.005$) (*Figure*2.7). Neither diploids or polyploids produced any seeds following self-pollination.

Field observations suggested that distylous *O. purpurea* populations were strongly self-fertile, as they successfully formed fruit capsules in these populations. As selfing experiments were done using only mid-morph plants, the self-compatibility of these could not be measured. However, based on the observed pollen size convergence in mid and short anther whorls (*Figure 2.5*) and because these populations are all monomorphic (only includes Long whorl plants), the formation of fruit strongly suggests that genetic self- and within-morph incompatibility systems have broken down in these populations.

Table 2.1: Summary of the effect of polyploidy on measured traits of *O. purpurea*. Effect sizes of WGD taken as significantly different from zero at p <0.05.

Trait	95% Confidence intervals	Effect size of polyploidy	Diploid size	p-value
Pollen (µm)	Can't Compute	1.072	56.024	0.0242*
Stomata Length(µm)	2.1761-6.1176	3.105	25.431	5.2e-08***
Epidermal Cell Area(μm^2)	1.2114-1.7038	1.430	1249.027	0.0001***
Petal Length(mm)	Can't Compute	2.958	19.730	0.0556
Petal Width (mm)	Can't Compute	1.127	11.495	0.0013**
Peduncle Length(mm)	0.5856-2.2330	3.212	11.588	0.5466
Peduncle-Bract(mm)	0.6204-2.0607	1.141	8.941	0.6830
Sepal Length (mm)	-0.0426-1.2225	0.580	5.896	0.2107
Sepal Width (mm)	-0.1017-0.3089	0.0415	0.899	0.5466
Bract Width (mm)	-0.0925-0.0490	-0.021	0.475	0.6830
Bract Length (mm	0.1244-1.5978	0.675	3.631	0.8645
Leaflet Length(mm)	Can't Compute	3.117	18.753	0.3500
Leaflet Width(mm)	0.9274-1.4283	1.152	18.716	0.3668
Leaflet Thickness(mm)	0.0293-0.1032	0.066	0.239	0.0154*
Clonal bulbil production (number of bulbils)	0.4495-1.2911	0.759	1.285	0.0001***
Bulb growth (2019-2020) (g)	0.0557-1.2339	0.649	0.665	0.0405*

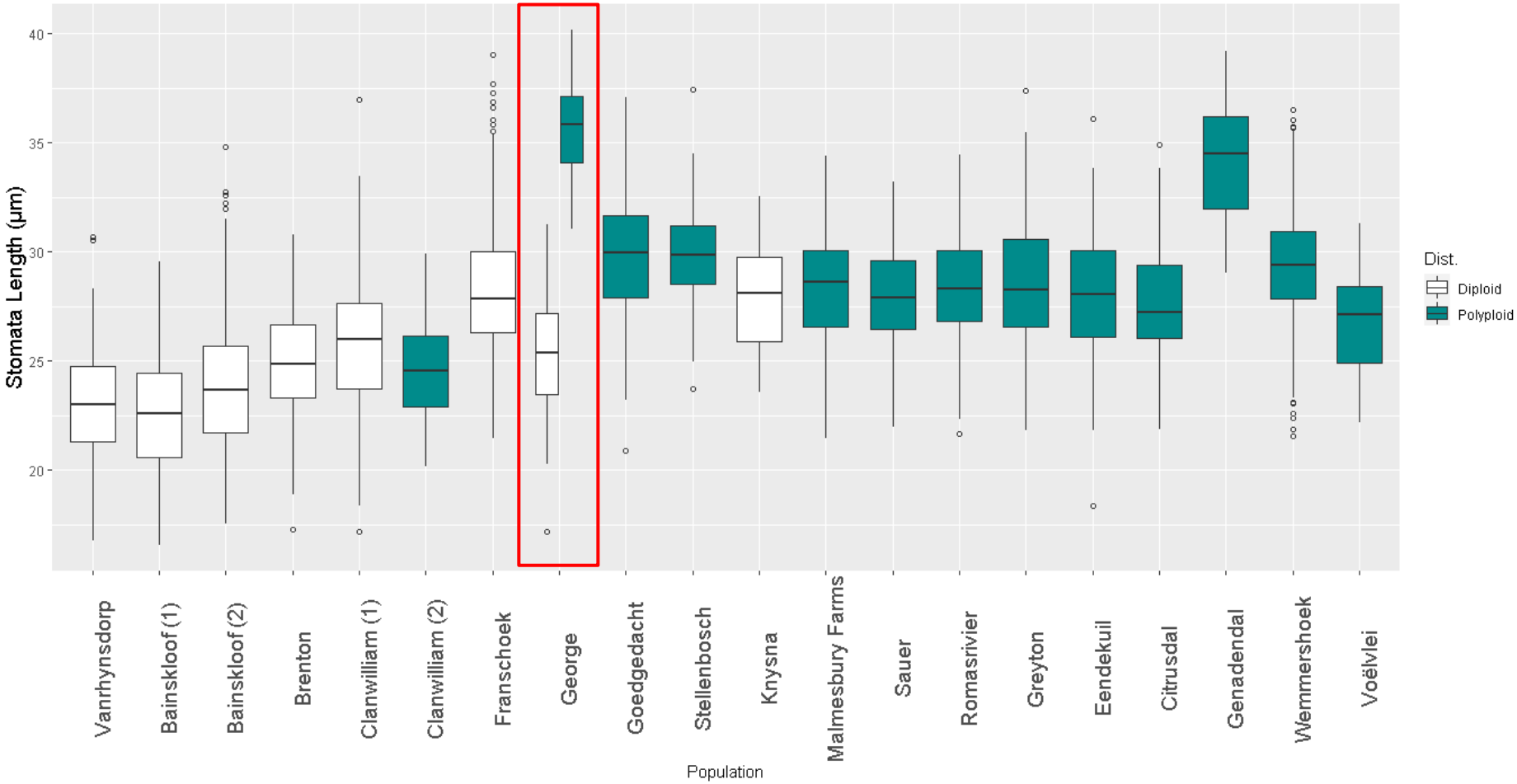

Figure 2.4: Stomata length distribution for *O. purpurea* diploids and polyploids. George was the only mixed-ploidy population containing diploids, that survived and flowered in cultivation. Polyploid stomata were, on average, significantly larger than diploid stomata (p =5.2e-08, *Table 2.1*)

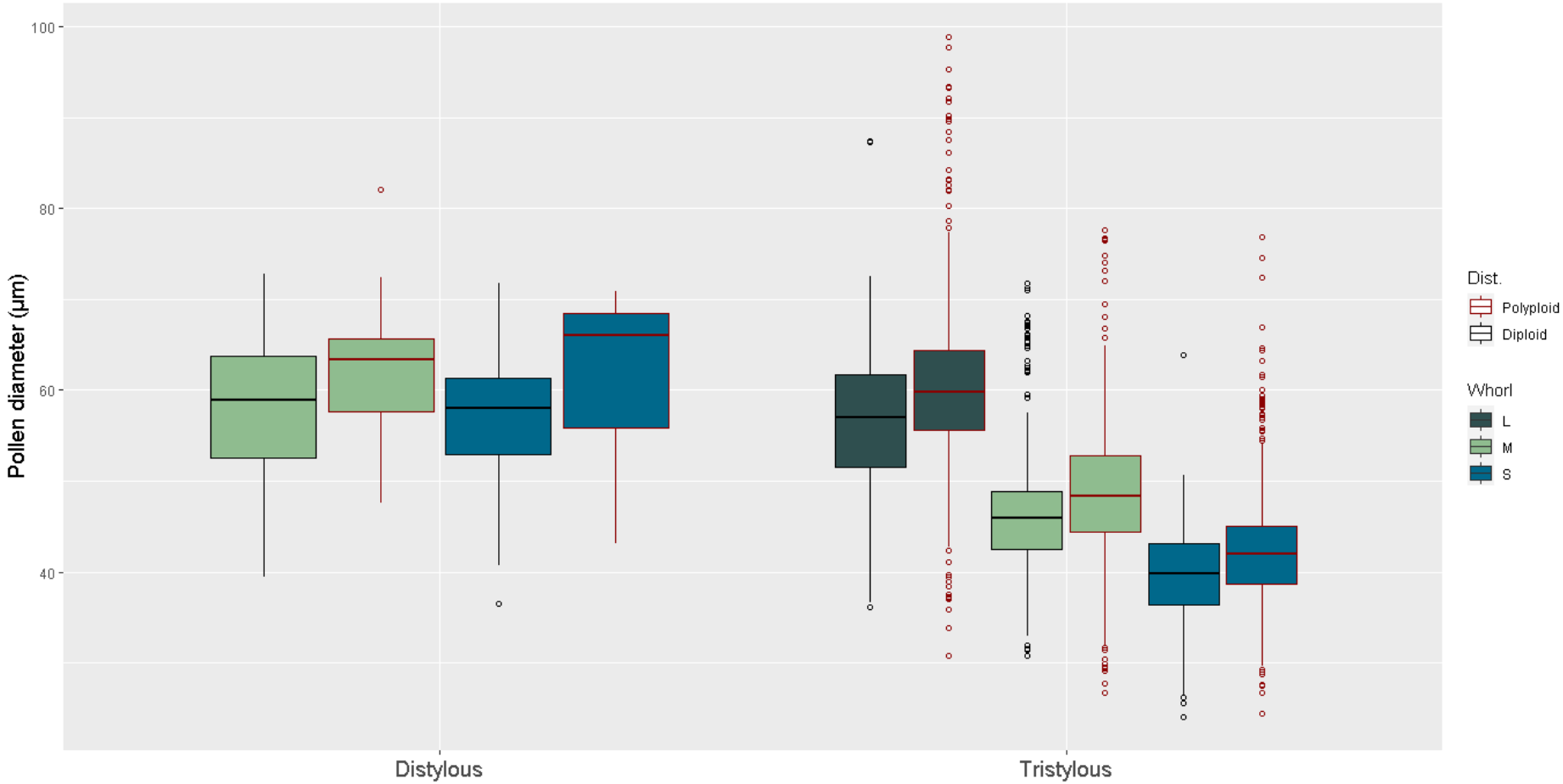

Figure 2.5: Size differences in pollen grain diameter per whorl (Long (L), Mid (M) and Short (S)) for *O. purpurea* diploid and polyploid populations for (left) distylous populations and (right) tristylous populations. Distylous polyploid measurements came from a single sample tetraploid co-occurring with distylous diploids.

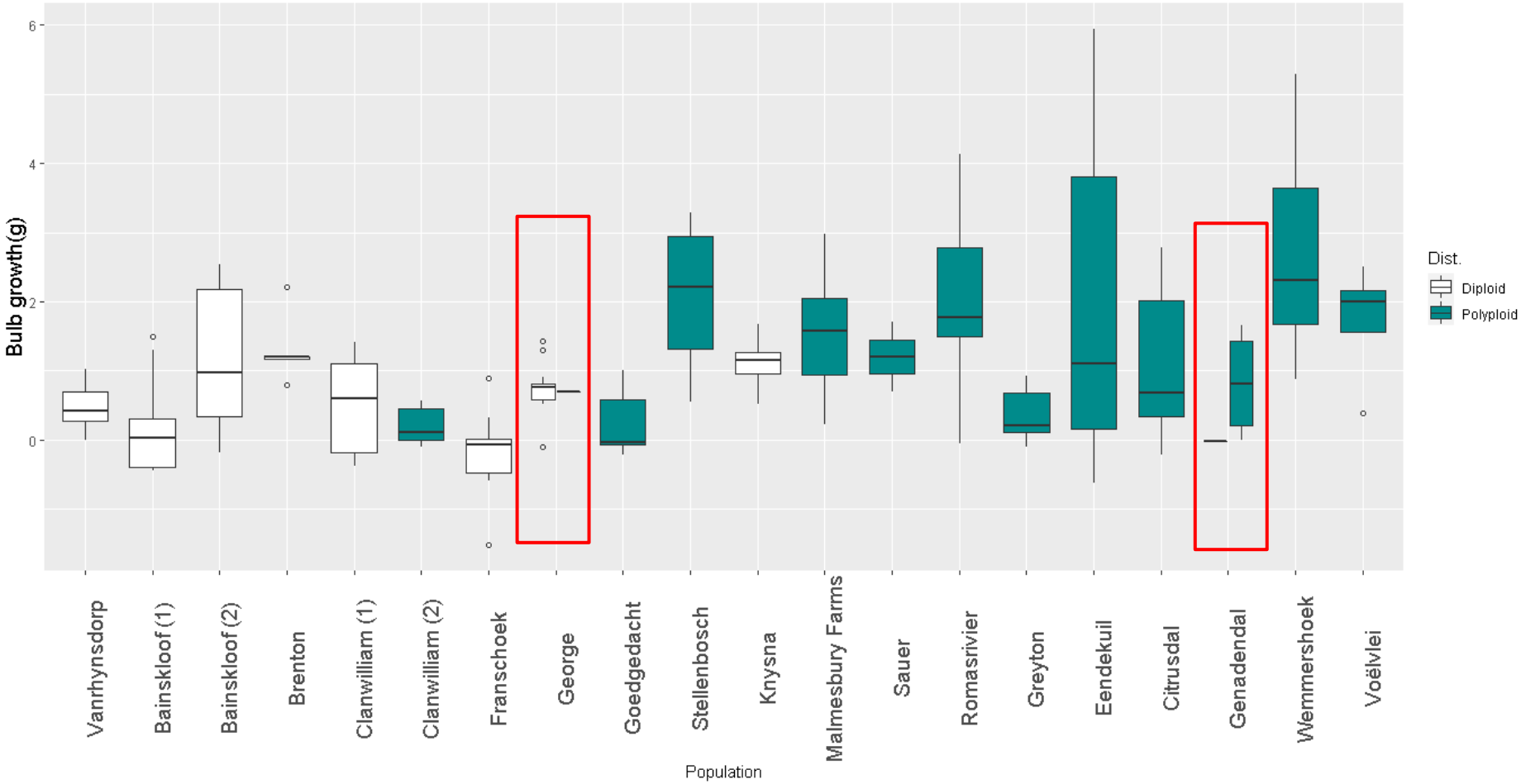

Figure 2.6: The increase in wet mass of *O. purpurea* bulbs (total mass of primary bulb and bulbils) across sampled populations between the period of March 2020 to December 2020 for diploids and polyploids. Red boxes indicate the only mixed diploid-polyploid populations sampled for bulb growth in this study. The George population had only a single polyploid, while the Genadendal population had only a single diploid.

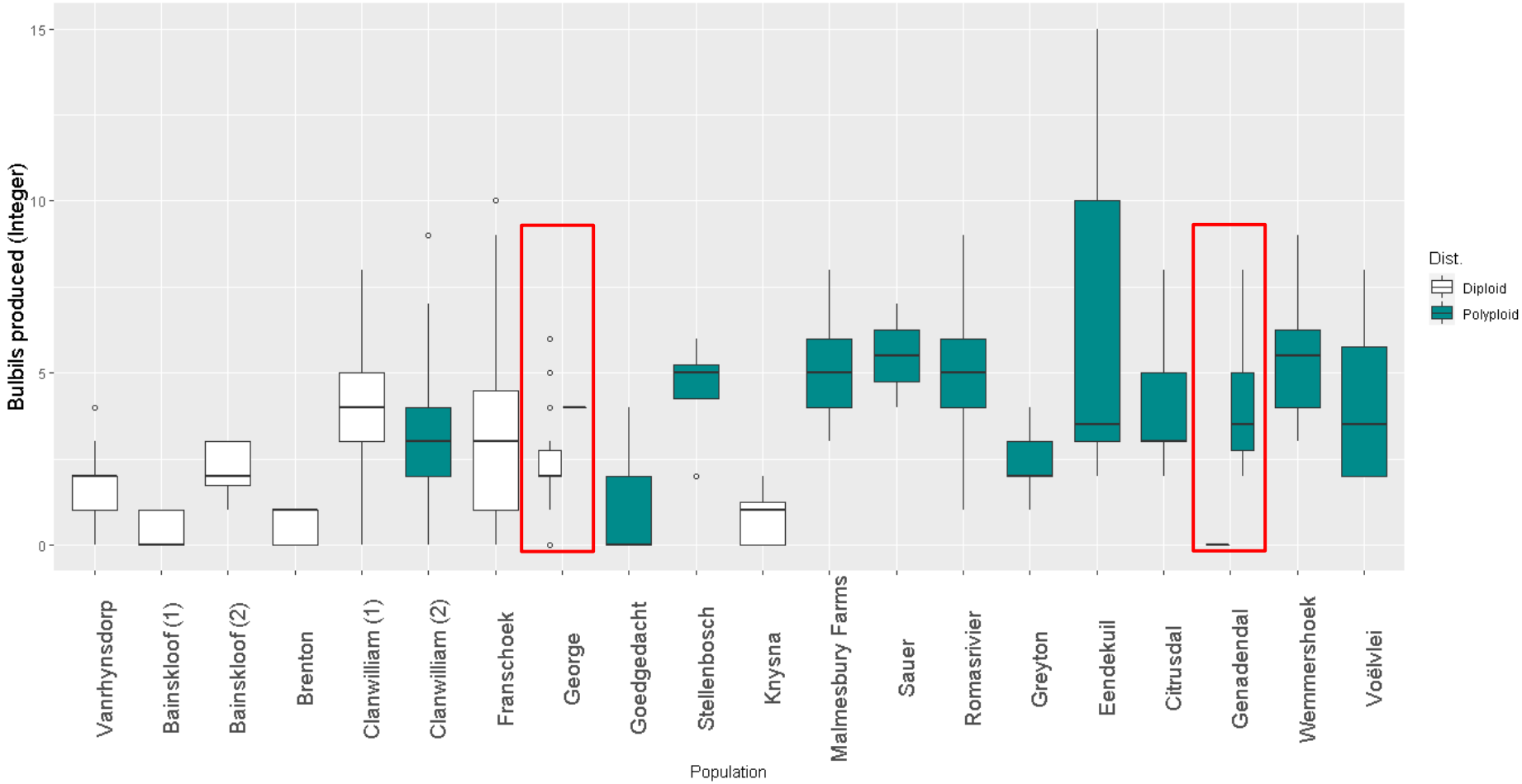

Figure 2.7: The number of bulbils formed in *Oxalis purpurea* during a single growing season from March 2020 to December 2020. Red boxes indicate the only mixed diploid-polyploid populations. The George population had only a single polyploid, while the Genadendal population only had a single diploid.

4. Discussion & Conclusions

WGD is expected to bring about enlargement of cells and organs with the effect size shown to remain consistent across multiple traits and measurement scales (Porturas *et al.* 2019). It is thought that plant cells enlarge (and cause organs and multi-cellular structures also to enlarge) as a direct result of more DNA present in the cell (Muntzing 1936, Stebbins 1971). There have, however, been notable exceptions to this pattern of cellular or higher-scale enlargement (Ning *et al.* 2009, Otto & Whitton 2000, Segraves & Thompson 1999, Vamosi *et al.* 2007), with some polyploids showing smaller organs than diploids (Trojak-Golcuh & Skomra 2013). Polyploids that do not show patterns of enlargement are thought to be in the minority though (Porturas *et al.* 2019).

Becker *et al.* (2020; Chapter 1) observed substantial variation in traits among both diploids and polyploids within individual *Oxalis* species. Polyploids differed significantly from diploids in stomata length, epidermal cell area and pollen grain diameter. The effect size, however, did not remain constant across taxa, nor did it remain consistent between measured traits as is predicted for most angiosperms. The present study explored whether this may be attributable to the relatively small per species sample sizes included by Becker *et al.* (2020; Chapter 1) by substantially increasing sample sizes in a single species, *O. purpurea*. Results presented here suggest the possibility of the Gigas effect being present among polyploids of this species at the cellular scale, but with very small effect sizes. Polyploid pollen grains a size increase of 2%, epidermal cells of 0.1% and stomata of 12%. These observed increases are substantially smaller than increase of 25% predicted for the Gigas among angiosperms (Porturas *et al.* 2019). Effect sizes was also inconsistent across traits, contrary to predictions of the Gigas effect remaining constant across traits. At the organ-level measurement scales, any significant effect is greatly decreased, with only two of twelve traits showing significant size increase for polyploids (leaflet thickness ($p = 0.0154$) and petal width ($p = 0.0013$)). Overall, with varying degrees of significance and varying effect sizes of polyploidy, I do not find convincing evidence to suggest a strong Gigas effect in *O. purpurea*.

The data suggests that trait sizes are not reliable predictors of polyploidy in *Oxalis*, especially with small sample sizes and in population-level comparisons, with individual populations often showing varying patterns in trait sizes to polyploidy. Given the unknown age of the multiple

WGD's in *Oxalis purpurea*, these results leave open the option that *Oxalis* neopolyploids indeed experience a temporary Gigas effect directly after formation, but that this may become diluted or lost due to subsequent independent adaptation and different evolutionary histories. This fits with the current evidence on the Gigas effect, which is often strongest in neopolyploids (Porturas *et al.* 2019, Soltis *et al.* 2015). This may be happening gradually as has been suggested in other lineages (Butterfass 1987, Oswald & Nuismer 2011, Ramsey 2011, Husband *et al.* 2016). Given that the two populations with co-occurring diploids and polyploids (George and Genadendal) mostly show very pronounced trait effects between diploids and polyploids but comparisons between other populations show a reduced effect, an increase in trait variation after WGD might be a more common occurrence than is currently thought. This, however, remains to be tested in *Oxalis* as these populations only contained one polyploid or one diploid in the entire sample, and we do not know the age of WGD events in this species.

The large variation in relative genome size for cytotypes are consistent with multiple origins for polyploids in *O. purpurea* (*Figure 2.2*). If *O. purpurea* polyploids originated from a single event we would expect to see similar relative genome sizes. This was not the case for *O. purpurea* polyploids. Majority of sampled polyploid populations contained no diploids (at least in the sample taken). This suggests that polyploids outcompete diploids after formation or have diverged significantly in niche occupation from diploids over time. Polyploids from mixed diploid-polyploid populations might be more recently formed than other established polyploid populations, since the Gigas effect may be more pronounced in neopolyploids and gets lost in subsequent generations through natural adaptation (Butterfass 1987, Oswald and Nuismer 2011, Ramsey 2011, Husband *et al.* 2016, Baduel *et al.* 2018). If this hypothesis is true, then the Gigas effect can only play a temporary and short-lived role in the success of polyploids in this system. It could help with initial establishment of a polyploid in a population, but the subsequent success of generations ultimately lies in local adaptation and possible hidden effects of polyploidization might then come into play. These possibilities are based on a very small sample size of one polyploid in a diploid population, and one diploid in a polyploid population, so I cannot make any claims around the expected natural variation in polyploid plants in this population. These possibilities could be supported if similar patterns were found with increased sampling.

Polyploid success might be attributed to an interactive effect between the Gigas and other ecological or genomic changes brought on by WGD (Levin 1983, Tate *et al.*, 2005). Polyploidy

has been linked to an increase in self-pollination, clonal reproduction and changes in growth rate (Van Drunen & Husband 2019). As a result, many invasive species are polyploids, suggesting that WGD may increase a plants invasive potential (Pandit *et al.* 2006, 2011). Since I do not find consistent evidence of the Gigas effect in *O. purpurea*, it raises the question why polyploids are still so frequently and abundantly found. *O. purpurea* polyploids were found to produce more and higher quality (larger) clonal propagules (*Figure 2.6, 2.7*). This is interesting, given that the above-ground traits measured in this study show highly variable evidence of a Gigas effect, so any potential increase in fitness inferred by the Gigas effect is not very strong. Polyploids have also been shown to have slower growth rates than diploids as a direct result of cell size increase (Gates 1909). Although we did not measure growth rate per se, *O. purpurea* polyploids should show some degree of higher metabolic activity than diploids to store more resources in the same amount of time. Our results might therefore reflect a hidden effect of ploidy on *O. purpurea* polyploid metabolism – an effect not brought on by enlargement of cells and organs. Another reason might be an uneven allocation of resources in polyploids, where polyploids store more resources in their bulbs rather than investing in above ground structures. This might explain in the inconsistency of the Gigas effect in leaf and flower traits.

WGD is associated with invasive *O. pes-caprae* L. (Krejčíková *et al.* 2013 (c), Castro *et al.* 2007, 2013, 2016), where only polyploid cytotypes are found in invasive ranges. The Gigas effect could be a valuable trait in polyploid invasiveness because of its associated changes to physiology, metabolism and environmental tolerance (Levin 1993, 2002, Warner & Edwards 1993, Coate *et al.* 2012, 2013, Wang *et al.* 2013). *O. purpurea* polyploids produce both larger and more clonal bulbils than diploids (*Figure 2.6* and 2.*7*) and can be seen as a way of increasing propagules, one of the prerequisites for invasiveness (Lockwood *et al.* 2009). There is, however, no clear link between invasive potential and the Gigas effect, suggesting that polyploidy may bring about ecological changes that increases a polyploids' fitness, without the Gigas effect playing a prominent role. To test whether polyploidy is indeed a driver of potential invasiveness, sampling cytotype distributions of *O. purpurea* populations in invasive ranges could provide insight into whether the invasive populations are solely polyploid. Furthermore, testing bulbil viability, survivability and spread in disturbed and undisturbed soils could also advise on the potential speed by which *O. purpurea* polyploids may spread in an introduced environment.

Although I found many mixed cytotype populations, these were mostly mixtures of polyploid cytotypes. Only three sampled populations contained diploids and polyploids in sympatry. These populations were mostly dominated by either polyploid or diploid cytotypes to the extent that from a sample of 12 plants only one plant was diploid or polyploid. Since cytotype and geographic distribution suggests that *O. purpurea* polyploids formed multiple times (given the variation in relative genome size and widespread and non-contiguous distribution of polyploids (Symonds *et al.* 2010)) this could also suggest that most polyploid populations were once diploid and after polyploid formation, diploids were locally outcompeted. Given these results it is interesting to find that diploids still occur just as abundantly as polyploids do. Since polyploids show a significantly higher bulbil production and size than diploids, they should effectively outcompete most diploid populations. This will happen because the amount of polyploid individuals are increased through clonal bulbil production. More individuals increase the chance that some would survive unfavorable conditions and persist. In cases where conditions are favorable, polyploids would quickly increase to become the dominant cytotype. Heavier bulbils also allow for a greater chance of survival during dormancy periods. This suggests that after every growing season more polyploids should survive the dry period and emerge during the following growing period. But polyploids are thought to do better in disturbed environments with intermediate climates (Knight *et al.* 2005, Stebbins 1971). It may be that the environmental heterogeneity of the Cape allows both polyploids and diploids to exist, each in specific microclimates, and each the dominant competitor in those areas. This might explain why we find so few co-occurring diploid-polyploid populations.

WGD is not just associated with an increase of vegetative clonal reproduction, but also increased self-pollination rates due to a breakdown in genetic incompatibility systems in polyploids (Richards 1997, Barringer 2007). Even with our small sample size of 20 plants, we were able to show that *O. purpurea* undergoes no or very low levels of self-pollination, regardless of ploidy level. This is interesting as genetic incompatibility systems of polyploids have been shown to break down, leading to an increase in self-pollination, which offers an escape from minority cytotype exclusion (Richards 1997, Levin 1975). It is possible that allopolyploids may experience higher levels of self-incompatibility breakdown than autopolyploids (Stebbins, 1950, 1971, Briggs & Walters 1997, Marfil *et al.* 2018).

Since *Oxalis* species show a low tendency to hybridize (du Preez *et al.* 2018; Salter 1944), *O. purpurea* polyploids are thought to be mostly autopolyploid. Ramsey & Schemske (2002)

reviewed the concept of lower self-compatibility in autopolyploids relative to allopolyploids and came to the conclusion that mostly, this is not the case. Even though the age of *O. purpurea* polyploids are unknown and I did not compare self-compatibility levels between allopolyploids and autopolyploids, the results suggest that polyploid *O. purpurea* plants show no increase in self-compatibility, as is suggested to accompany WGD. If *O. purpurea* allopolyploids are found and show higher selfing levels, then mostly autopolyploid formation could explain the low self-compatibility in *O. purpurea* polyploids. A more likely explanation, however, is tristyly. Tristyly is a mechanism which enforces outcrossing and genetic self-incompatibility (Barrett 2002, Barrett 1992). Therefore, maintenance of tristyly in *Oxalis* may explain why *O. purpurea* polyploids show such low levels of self-compatibility. This is also supported by the fact that distylous populations of *O. purpurea* formed seed-bearing fruit through self-fertilization (F.W. Becker, field observations) (Trelease 1882, Weller *et al.* 2007, Zietsman 2007). As a result, the only major avenue for polyploids to avoid minority cytotype exclusion is in forming more and larger clonal bulbils.

In conclusion, *O. purpurea* polyploids show some evidence for the Gigas effect at the cellular scale, albeit a rather small and inconsistent effect, while any polyploidy-related increase in size is mostly, if not entirely, lost at larger scales. The variation of effect sizes measured at population level indicates that the Gigas effect is not able to provide a reliable and accurate predictor to distinguish between polyploids and diploids in field populations of *Oxalis purpurea*. There were no differences in self-pollination between cytotypes, suggesting that WGD does not facilitate the breakdown of self-incompatibility in *O. purpurea*. Polyploidy does, however, increase invasive potential in *O. purpurea* polyploids via increased below-ground allocation of resources compared to diploids. WGD may therefore bring about fitness advantages to ecological traits in *O. purpurea* by means other than the Gigas effect.

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General Conclusions

The Gigas effect is expected to show a prominent increase in the size of traits (20 -25%) in flowering plants and be consistent across measured traits (Porturas *et al.* 2019). I found, both across species and extensive sampling within *O. purpurea*, that this is not supported for *Oxalis* polyploids. The size-related effect of polyploidy was very small, ranging from a 10% increase in stomata lengths to 0.1% in epidermal cell area. The polyploid trait responses were also inconsistent across and within species and between populations of *O. purpurea*, meaning that the Gigas effect is not a reliable predictor of ploidy level in *Oxalis*. Previous authors have also reported considerable variation in trait responses (Ning *et al.* 2009, Otto & Whitton 2000, Vamosi *et al.* 2007). Segraves & Thompson (1999) found a significant increase in trait size across all polyploid individuals relative to diploid individuals, but with considerable variation in floral morphology. The variation was attributed to different ages of polyploids and large geographic scale of sampling. Since *O. purpurea* polyploids show indistinct 2C DNA values, especially at higher ploidy cytotypes (tetra- penta- and hexaploids), I suspect that *Oxalis* polyploids to originate from recurrent and multiple formations. This would be one possible explanation for the variability I observed in *Oxalis.* Another possibility is that, given the different ages of the polyploids I studied, post WGD adaptation in the heterogenous Cape environment may overshadow the Gigas effect over evolutionary time. The few sampled sympatric diploid/polyploid populations showed the most distinct differences between traits. Given the small and inconsistent effects observed, the Gigas effect probably plays a minor role in increasing *Oxalis* polyploid fitness in the long run.

O. purpurea polyploids had significantly larger and more bulbils than diploids, meaning that WGD may have a hidden effect on clonal reproduction and resource storage. The expected increased levels of self-compatibility in polyploids was not observed in *O. purpurea*. Together these finding suggest that *Oxalis* polyploids may exhibit an uneven partitioning of what little Gigas effect is observed and that it may be more pronounced in below-ground structures. WGD therefore still confers a fitness advantage to polyploids through increased vegetative clonality. This may explain the success of *Oxalis* species, and in particular *O. purpurea*, in invading new areas.

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